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THE ROLE OF BIOACTIVE LIPID MEDIATORS AND EXTRACELLULAR VESICLES IN MESENCHYMAL STROMAL CELL IMMUNOMODULATION

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ACADEMIC DISSERTATION

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*Equal contribution

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1 Author designed the study, performed the cell culture, flow cytometric and mass spectrometric experiments, participated in setting up the LC-MS/MS method, analysed the data and participated in the manuscript writing.

2 Author designed the study, performed the cell culture and mass spectrometric experiments, analysed the data and wrote the manuscript.

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Tigistu-Sahle F, Lampinen M, Kilpinen L, **Holopainen M**, Lehenkari P, Laitinen S, Käkelä R (2017) Metabolism and phospholipid assembly of polyunsaturated fatty acids in human bone marrow mesenchymal stromal cells. Journal of Lipid Research 58: 92-110

ABBREVIATIONS

AA	Arachidonic acid
ALA	α-Linolenic acid
BLT	B leukotriene receptor
CFSE	Carboxyfluorescein succinimidyl ester
COVID-19	Coronavirus-induced disease 2019
COX	Cyclooxygenase
CYP450	Cytochrome P450
CysLT	Cysteinyl leukotriene receptor
DGLA	Dihomo-γ linolenic acid
DHA	Docosahexaenoic acid
DHRS9	Dehydrogenase/reductase 9
DPA	Docosapentaenoic acid
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
ESI-MS/MS	Electrospray ionisation-tandem mass spectrometry
EV	Extracellular vesicle
FBS	Foetal bovine serum
GC	Gas chromatography
GP	Glycerophospholipid
GvHD	Graft-versus-host disease
hBMSC	Human bone-marrow derived mesenchymal stromal cell
HDHA	Hydroxydocosahexaenoic acid
HDPA	Hydroxydocosapentaenoic acid
HEPE	Hydroxyeicosapentaenoic acid
HETE	Hydroxyeicosatetraenoic acid
HpDHA	Hydroperoxydocosahexaenoic acid
HpDPA	Hydroperoxydocosapentaenoic acid
HpETE	Hydroperoxyeicosatetraenoic acid
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon γ
IL	Interleukin
ISCT	International Society of Cell and Gene Therapy
ISEV	International Society for Extracellular Vesicles
LA	Linoleic acid
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LM	Lipid mediator
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LT	Leukotriene
LXA_4	Lipoxin A_4
LXB_4	Lipoxin B_4

MaR1	Maresin 1
MaR2	Maresin 2
$MaR_{n\text{-}3\ DPA}$	n-3 DPA-derived maresin
M-CSF	Macrophage colony-stimulating factor
MCTR	Maresin conjugates in tissue regeneration
MEM	Minimum essential medium
MerTK	Mer receptor tyrosine kinase
miRNA	microRNA
MMP-2	Matrix metalloproteinase 2
Mreg	Regulatory macrophage
MSC	Mesenchymal stromal cell
MUFA	Monounsaturated fatty acid
NK cell	Natural killer cell
NTA	Nanoparticle tracking analysis
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PCA	Principal component analysis
PCTR	Protectin conjugates in tissue regeneration
PD1	Protectin D1
PD-L1	Programmed death-ligand 1
$PD_{n\text{-}3\ DPA}$	n-3 DPA-derived protectin
PE	Phosphatidylethanolamine
PG	Prostaglandin
PI	Phosphatidylinositol
PL	Phospholipid
PLA_2	Phospholipase A_2
PMN	Polymorphonuclear cell
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
QPCR	Quantitative polymerase chain reaction
RCTR	Resolvin conjugates in tissue regeneration
RvD	D-series resolvin
$RvD_{n\text{-}3\ DPA}$	n-3 DPA-derived D-series resolvin
RvE	E-series resolvin
RvT	13-series resolvin
SM	Sphingomyelin
SPM	Specialized proresolving mediator
TGF-β1	Transforming growth factor β1
Th	CD4+ T helper cell
TNF-α	Tumour necrosis factor α
Treg	Regulatory T cell
TSG-6	Tumor necrosis factor-stimulated gene 6
TXA_2	Thromboxane A_2
TXB_2	Thromboxane B_2

ABSTRACT

Human mesenchymal stromal cells (hMSCs) are fibroblast-like cells that have an exceptional ability to modulate immune cells. Due to their immunomodulatory properties, hMSCs have been employed in various clinical trials in the treatment of autoimmune or inflammatory diseases. Even though hMSC therapy has yielded multiple promising results, not all trials have been successful. Due to the discrepancies in the therapeutic response, hMSC therapy requires further development and standardisation.

While optimisation of the culture conditions provides one method to improve the therapeutic efficacy of hMSCs, fine-tuning the culture conditions requires deep understanding of the hMSC immunomodulatory mechanisms. It has been established that hMSCs mediate their therapeutic effect via cell-cell contact and especially by secreting several paracrine factors that include lipid mediators and extracellular vesicles (EVs). Intriguingly, hMSC-derived EVs (hMSC-EVs) are able to mediate the therapeutic response of MSCs, which has led to a growing interest in and research on cell-free hMSC-EV therapy.

In addition to EVs, lipid metabolism and especially the lipid mediator prostaglandin E_2 (PGE_2) are vital to the hMSC immunomodulation. Interestingly, the membrane lipid composition correlates with the immunosuppressive capacity of hMSCs, suggesting that membrane lipids play a role in mediating the immunomodulatory response of hMSCs. Lipid mediators are derived from polyunsaturated fatty acids (PUFAs) that can be stored in membrane phospholipids. Over the past few decades, the significance of PUFA-derived lipid mediators in orchestrating the dampening phase of inflammation, i.e., resolution, has been unravelled. These novel specialized proresolving mediators (SPMs) have several essential roles in modulating immune cells, including macrophages.

The role of these SPMs in hMSC immunomodulation has received only marginal interest, and whether hMSCs produce multiple SPMs has not been determined. Keeping in mind that hMSC-EVs mediate the therapeutic response of MSCs, we investigated the lipid metabolism of hMSCs and their EVs after supplementing the cells with lipid mediator precursor PUFAs: arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid (DHA). Due to the supplementation, the membrane phospholipid and fatty acid composition were notably modified and the downstream lipid mediator production was enhanced. Excitingly, we were able to demonstrate for the first time that hMSCs produce several SPMs, which may mediate the immunomodulatory response of hMSCs. Remarkably, we showed that the PUFA modifications in the phospholipid composition were transferred into the EVs, highlighting the importance of EVs as transporters of the immunomodulatory factors derived from hMSCs.

Because of their importance in the resolution of inflammation, we investigated the effects of hMSCs and hMSC-EVs on macrophages with a focus on regulatory macrophages (Mregs). We elucidated that both hMSCs and hMSC-EVs enhanced the anti-inflammatory and proresolving properties of these less-studied macrophages, highlighting the importance of PGE_2 in the function of hMSC-EVs. Moreover, the hMSC secretome increased the CD206 expression and *Candida albicans* phagocytosis activity of macrophages, demonstrating a functional change in the macrophage properties. Fascinatingly, the DHA-supplemented hMSCs even further skewed the macrophage phenotype in an anti-inflammatory direction. However, the change was limited, and the elucidation of the functional effects of PUFA supplementations on hMSCs require additional investigations.

In conclusion, this thesis provides further evidence that the lipid metabolism has an essential role in hMSC functionality and that the SPM production may represent an additional mechanism in hMSC immunomodulation. For the first time, we have explored the impact of hMSCs and hMSC-EVs on Mregs and our results highlight the importance of EVs as the mediators of hMSC immunomodulation. Furthermore, we investigated the possibility of improving the hMSC immunomodulation with PUFA supplementations that would represent an easy and safe way to enhance the therapeutic potential of hMSCs or hMSC-EVs. A more detailed understanding of the complex immunomodulatory mechanisms of hMSCs is in key position when investigating new possibilities in the development of hMSC therapy.

1 INTRODUCTION

The field of cell therapy has great potential in the treatment of various diseases with no known cure or insufficient options available. The medical applications for cell therapy are typically related to immunological diseases and regenerative medicine. Cell therapy approaches include the utilisation of genetically modified immune cells, such as chimeric antigen receptor T cells to combat acute lymphoblastic leukaemia (June et al. 2018), induced pluripotent stem cells in regenerative medicine, such as in the treatment of age-related macular degeneration (Shi et al. 2017), or cultured primary cells, such as mesenchymal stromal cells (MSCs) that are employed in both regenerative and immunological indications (Le Blanc and Mougiakakos 2012; English 2013). In addition to the MSCs, MSC-derived extracellular vesicles (EVs), small membrane particles with immunomodulative properties, show great promise in several animal models and also in the clinic (Giebel et al. 2017).

MSCs are intriguing cells with regenerative abilities in addition to a capacity to modulate immune cells, mainly towards anti-inflammatory direction, and thus, they have been utilised in hundreds of clinical trials (Noronha et al., 2019; https://clinicaltrials.gov). Even though multiple reports have described the positive effects of MSC therapy, consistent and reproducible results from the clinic remain to be achieved (Galipeau and Sensébé 2018). Thus, there is a need to improve MSC therapy further, and the more recently elucidated MSC-EV therapy requires further development and standardisation (Giebel et al. 2017).

The effects of various external factors, such as inflammatory cytokines, hypoxia and polyunsaturated fatty acids (PUFAs), have been investigated as methods to prime MSCs and to improve their therapeutic efficacy (Chatgilialoglu et al. 2017; Abreu et al. 2018; Noronha et al. 2019). Interestingly, PUFAs are precursors for several lipid mediators (LMs), that play key roles in the induction and dampening of inflammation (Serhan 2017). The active dampening phase is called the resolution of inflammation, which is regulated by multiple proresolving LMs. These highly bioactive LMs have beneficial effects in multiple disease models of inflammatory diseases (Marcheselli et al. 2003; Haworth et al. 2008; Spite et al. 2009). Recently, research on MSC immunomodulation and its connection to lipid metabolism has evoked increasing interest (Fang et al. 2015; Tsoyi et al. 2016; Tigistu-Sahle et al. 2017; Abreu et al. 2018). The reported results highlight the need for further investigations to elucidate the immunomodulatory mechanisms of action of MSCs and MSC-EVs related to lipids and LMs. With increasing understanding of the immunomodulation of MSCs and MSC-EVs, we can discover ways to improve their therapeutic efficacy.

2 REVIEW OF THE LITERATURE

2.1 MESENCHYMAL STROMAL CELLS

MSCs are multifunctional cells with immunomodulatory and regenerative properties and they show great promise in the field of cell therapy. Friedenstein and colleagues first discovered these fibroblast-like cells from bone marrow in the 1970s (Friedenstein et al. 1968). Subsequent to the observations indicating that these cells are capable of differentiating into mesodermal lineages (Caplan 1991) and have the ability to modulate immune cells (Bartholomew et al. 2002; Di Nicola et al. 2002), MSCs attracted wide interest in the field of regenerative and immunological cell therapy. These cells were termed "mesenchymal stem cells", however, it was later observed that the MSC population isolated from tissue is rather heterogeneous. Furthermore, the data supporting the stemness of the plastic-adhered cultured MSCs was not convincing. Hence, it was suggested by the International Society of Cell and Gene Therapy (ISCT) that MSCs should be referred to as "mesenchymal stromal cells", which better describes the nature of this cultured and heterogeneous cell population (Horwitz et al. 2005).

Even though MSCs are found in many tissues around the body, MSCs *in vivo* are relatively rare (Le Blanc and Mougiakakos 2012). Typical sources for *ex vivo* culture of MSCs include bone marrow, adipose tissue and, to a lesser extent, umbilical cord blood and placental cells. All of the aforementioned are adult tissue sources with relatively easy accessibility. Yet, adipose tissue provides the most convenient and the least invasive source for MSC extraction from healthy and voluntary donors, also yielding most MSCs per gram of tissue (Strioga et al. 2012).

MSCs derived from different donors and tissue sources have distinct characteristics, including a varied cytokine profile, differentiation and immunomodulatory potential (Kilroy et al. 2007; Alt et al. 2012; Strioga et al. 2012). To better standardise the cells, ISCT has presented the minimal criteria required for MSC characterisation (Dominici et al. 2006). These criteria include adherence to plastic, the expression of cell surface markers CD73, CD90 and CD105, and a lack of expression of hematopoietic markers CD11b, CD14, CD19, CD34, CD45, CD79a and HLA-DR. Even so, it has been shown that clinical MSCs may express HLA-DR without it affecting their phenotype or functionality *in vitro* (Grau-Vorster et al. 2019). Additionally, MSCs need to elicit multipotent differentiation potential by differentiating into osteoblasts, adipocytes and chondroblasts. When utilised in immunological indications, it has been suggested to test the immunomodulatory potential of each MSC culture (Krampera et al. 2013). However, an *in vitro* study showed that the immunomodulatory potential of MSCs did not correlate with their

therapeutic outcome, indicating that these tests are not good indicators for the therapeutic potential of MSCs (von Bahr et al. 2012b).

The wide interest on MSC therapy arises from these cells' regenerative abilities and, most importantly, their intriguing ability to modulate the phenotype and physiology of various immune cells (described in more detail in **Section 2.1.1**) (Le Blanc and Mougiakakos 2012; English 2013). One great advantage of MSC-based therapy is the possibility to use allogeneic cells thanks to their immune privileged, or rather immune evasive, behaviour (Ankrum et al. 2014). Utilisation of allogeneic MSCs has advantages over the use of autologous cells, including the pre-banking of fully characterised cells, which makes it possible to treat the patient quickly without a time-consuming expansion period (Galipeau and Sensébé 2018). Although most of the reported effects of MSCs are anti-inflammatory or immunosuppressive, proinflammatory effects have also been reported (Waterman et al. 2010). It seems likely that the direction of the MSC immunomodulatory response is determined by the inflammatory cues of the microenvironment. For example, human adipose tissue-derived MSCs from obese or diabetic donors exhibit a more pronounced inflammatory response than MSCs derived from lean donors due to the upregulated inflammatory state of the adipose tissue in the former groups (Serena et al. 2016). Interestingly, priming, also known as licencing, of MSCs with proinflammatory cytokines, such as interferon γ (IFN-γ) and tumour necrosis factor α (TNF-α), *in vitro* improves their immunomodulatory properties as MSCs ready themselves to counteract inflammation (Krampera et al. 2006; François et al. 2009; Waterman et al. 2010; Krampera 2011).

The therapeutic potential of MSCs is derived from cell-cell contact-dependent and soluble paracrine factors, with the latter giving rise to most of the immunomodulatory effects of MSCs (English 2013). The effects of the soluble factors are largely mediated by EVs that the cells secrete (Giebel et al. 2017). Remarkably, MSC-derived EVs (MSC-EVs) can elicit a similar therapeutic response to the cells, which has raised interest towards cell-free MSC therapy (Bruno et al. 2009; Lai et al. 2010; Zhang et al. 2014). While the complete picture of the MSC mechanisms of action has yet to be established, the deepening of the knowledge of MSC immunomodulation plays a main role in finding ways to improve MSC therapy.

2.1.1 IMMUNOMODULATORY MECHANISMS

The immune response mediated by the adaptive and the innate immune system is essential for the normal functioning of the body. However, dysregulation in the functioning of immune cells is related to the pathogenicity of multiple diseases. MSCs have an intriguing ability to modulate immune cells, thereby inducing changes in their function and phenotype, which is the key foundation of MSC-based therapies. Several mechanisms by which MSCs

induce these immunomodulatory, and often anti-inflammatory and immunosuppressive, changes have been unravelled. These effects are mediated via cell-cell contact and soluble molecules, such as indoleamine 2,3-dioxygenase (IDO), prostaglandin (PG)E_2, ecto-5′-nucleotidase, tumour necrosis factor-stimulated gene 6 (TSG-6), transforming growth factor β1 (TGF-β1), microRNAs (miRNAs) and numerous other molecules depicted in **Figure 1** (Volarevic et al. 2017; Song et al. 2020).

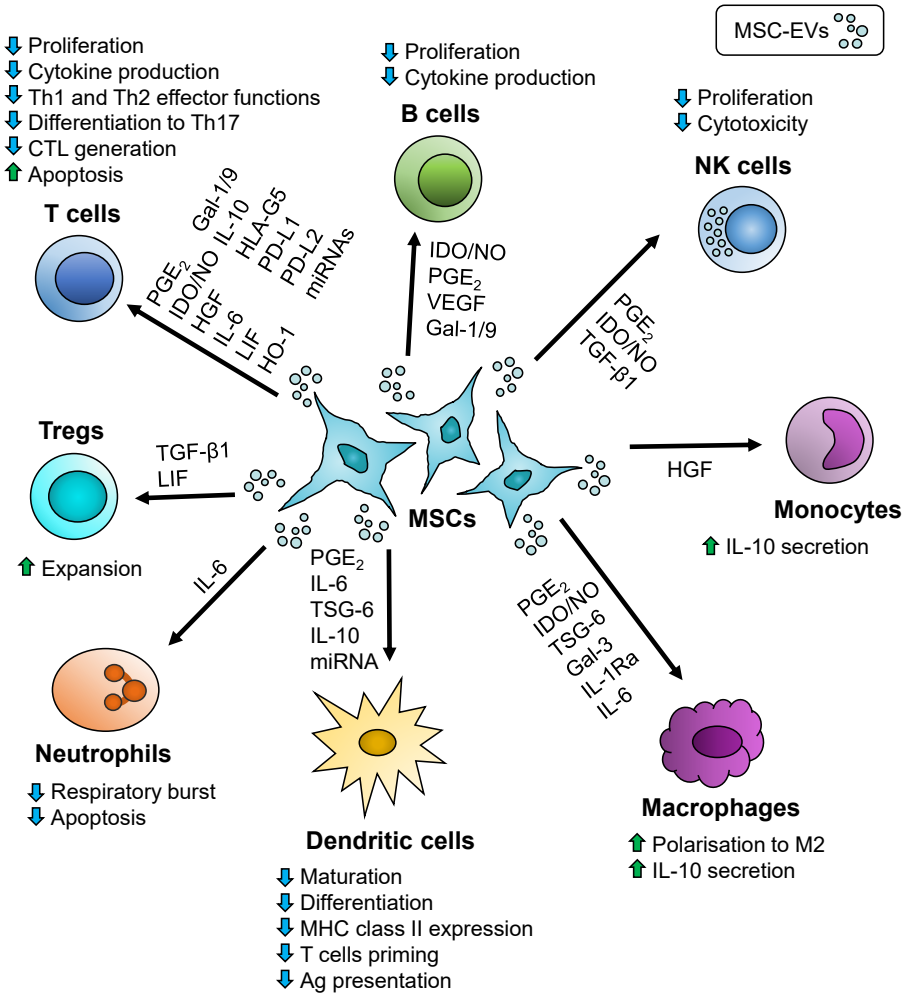

Figure 1 **Immunomodulatory mechanisms of MSCs.** Ag, antigen; CTL, CD8+ cytotoxic T-lymphocyte; Gal, galectin; HGF, hepatocyte growth factor; HLA-G5, human leukocyte antigen-G5; HO-1, heme oxygenase-1; IDO, indoleamine 2,3-dioxygenase; IL, interleukin; IL-1Ra, interleukin 1 receptor antagonist; LIF, leukaemia inhibitory factor; MHC, major histocompatibility complex; miRNA, microRNA; MSC-EVs, mesenchymal stromal cell-derived extracellular vesicles; NK cell, natural killer cell; NO, nitric oxide; PD-L, programmed death ligand; PGE_2, prostaglandin E_2; TGF-β1, transforming growth factor β1; Th, T helper cell; Treg, regulatory T-lymphocyte; TSG-6, tumour necrosis factor-stimulated gene 6.

One of the main soluble factors mediating MSC immunomodulation is the tryptophan-catabolizing enzyme IDO. This soluble enzyme degrades the essential amino acid tryptophan into kynurenine and other metabolites (Mellor and Munn 1999). Tryptophan deprivation directly inhibits T-, B- and natural killer (NK)-cell proliferation, and kynurenine with its metabolites may induce the apoptosis of T cells (Volarevic et al. 2017). IDO is expressed by human MSCs, while murine MSCs produce nitric oxide to achieve a similar immunosuppressive effect (Meisel et al. 2004; Ren et al. 2009). Nitric oxide is produced by inducible nitric oxide synthase, the expression of which is induced by proinflammatory cytokines.

PGE_2 is a LM derived from arachidonic acid (AA) and it has traditionally been considered to be a proinflammatory molecule. However, PGE_2 is produced by MSCs constitutively (also induced by proinflammatory stimuli) and it has multiple anti-inflammatory functions, e.g., mediating the polarisation of macrophages towards an anti-inflammatory phenotype and suppressing T-cell activation and polarisation (Aggarwal and Pittenger 2005; English et al. 2007; Zhang et al. 2010; Ylöstalo et al. 2012). The effects of MSC-derived PGE_2 are mediated via EP4 and possibly EP2 receptors (Németh et al. 2009; Duffy et al. 2011).

Ecto-5′-nucleotidase, also known as CD73, is expressed on MSCs and MSC-EVs, and it converts AMP to adenosine (Saldanha-Araujo et al. 2011; Amarnath et al. 2015; Kerkelä et al. 2016). Adenosine is an immunosuppressive molecule that regulates inflammation mainly by counteracting the function of proinflammatory ATP (Sitkovsky et al. 2004).

miRNAs are small non-coding RNAs that typically consist of 22 nucleotides and regulate post-transcriptional gene expression (Kroesen et al. 2015). MSCs and especially MSC-EVs contain several miRNAs, including also miRNAs that regulate the functioning of immune cells and mediate the therapeutic response of MSC-EVs (Vallabhaneni et al. 2015; Kilpinen et al. 2016; Deng et al. 2018; Shojaati et al. 2019).

MSCs exert their actions also through cell-cell contact-dependent mechanisms. The known mechanisms include programmed death-ligand 1 (PD-L1)-mediated effector T-cell suppression (Augello et al. 2005; Davies et al. 2017), FAS ligand-induced apoptosis of T cells (Akiyama et al. 2012) and cell adhesion molecules ICAM-1 and VCAM-1-mediated immunosuppression (Ren et al. 2010). Intriguingly, MSCs are known to transfer mitochondria to macrophages via tunnelling nanotubes that increases the phagocytic activity of macrophages (Jackson et al. 2016).

Additionally, the apoptosis of MSCs and their subsequent efferocytosis, i.e., the clearance of apoptotic cells, has been indicated to mediate MSC therapeutic response *in vivo* (Galleu et al. 2017). When administered intravenously or intra-arterially, MSCs are typically trapped in the lungs and many of them are phagocytosed relatively quickly (Gao et al. 2001; Schrepfer et al. 2007; Németh et al. 2009). Regardless of the trapping, the MSC administration can induce protective effects in distant tissues, such as the

heart, due to the effect of the secreted soluble factors and EVs transporting them (Lee et al. 2009; Lai et al. 2010). Fascinatingly, by inhibiting the EV production of MSCs, the therapeutic effects of MSCs are attenuated, highlighting the important role of EVs in MSC functioning (Di Trapani et al. 2016; Kim et al. 2019). In fact, the majority of MSC-derived immunomodulatory effects are most likely mediated by EVs, which have an essential role in cellular communication (Giebel et al. 2017).

2.1.1.1 Extracellular vesicles

EVs are small bilayered membrane particles secreted by all cell types. EVs have a crucial role in intracellular communication and affect both physiological and pathophysiological signalling (Yáñez-Mó et al. 2015). EVs carry receptors, proteins, RNAs, lipids and LMs as cargo that they transfer from the sending cell to the receiving cell (Subra et al. 2010; Yáñez-Mó et al. 2015; van Niel et al. 2018; Valkonen et al. 2019). The importance of EVs was highlighted by Raposo and colleagues in 1996, when it was discovered that EVs exert immunological functions and they have since attracted interest as therapeutic agents and drug-carrying vehicles (Raposo et al. 1996; Lener et al. 2015; Vader et al. 2016).

EVs are categorised into two main classes: exosomes and microvesicles (microparticles) depicted in **Figure 2** (van Niel et al. 2018). Exosomes are formed via an endosomal pathway and released in the fusion of the late endosome or multivesicular body with the plasma membrane. Microvesicles are secreted from the plasma membrane by fission, budding or shedding. The EV size range varies from 50 to 150 nm in exosomes and from 50 nm to up to 1 μm in microvesicles. Recently, with the development of analytical tools to detect EVs, a new group of nanoparticles with the size of ~35 nm, called exomers, has been identified (Zhang et al. 2018). In the literature, EVs are referred to by multiple other names based on their origin or function, such as apoptotic bodies shed during apoptosis, oncosomes produced by cancer cells and migrasomes transporting cargo during cell migration (van Niel et al. 2018).

Due to the overlapping size ranges and similar morphology, exosomes and microvesicles cannot be separated from each other based on size alone. Different class-specific markers have been proposed, such as CD63 for exosomes, however, these markers are not entirely specific and cannot be used as categorising factors (Bobrie et al. 2012). Thus, the International Society for Extracellular Vesicles (ISEV) has published minimal information suggestions that urge researchers to refer to the investigated vesicles with the general term EVs and to characterise the isolated EVs (size, morphology and semi-quantitative protein analysis) before class-specific identification tools are developed (Lötvall et al. 2014; Théry et al. 2018).

MSCs secrete immunologically active EVs, which can induce a similar immunomodulatory response as the cells (Bruno et al. 2009; Lai et al. 2010;

Zhang et al. 2014). MSC-EVs contain various lipids, fatty acids and proteins, such as phospholipases, fatty acid binding proteins, Rab-proteins, annexins and heat shock proteins (Kilpinen et al. 2013a; Haraszti et al. 2016; van Balkom et al. 2019). Moreover, MSC-EVs contain PGs, cytokines, growth factors, ecto-5′-nucleotidase and multiple miRNAs, which mediate the therapeutic response of MSCs (Deng et al. 2018). For example, the immunomodulatory TSG-6, TGF-β1, PD-L1 and galectin-1 are transported by MSC-EVs (Lee et al. 2009; Mokarizadeh et al. 2012; Kim et al. 2020).

The composition of EVs varies according to the tissue source, activation state and the EV isolation method (Yáñez-Mó et al. 2015; Brennan et al. 2020). Accordingly, the composition of MSC-EVs derived from different tissue sources is heterogeneous and the content can be further altered via the activation of MSCs (Kilpinen et al. 2013a; Börger et al. 2017). However, MSC-EVs derived from different tissue sources and isolated with multiple methods share common features in their proteomic signature (van Balkom et al. 2019). These results suggest that these EVs have unique properties that may be employed in the standardisation of therapeutic MSC-EVs and to unravel their immunomodulatory mechanisms of action.

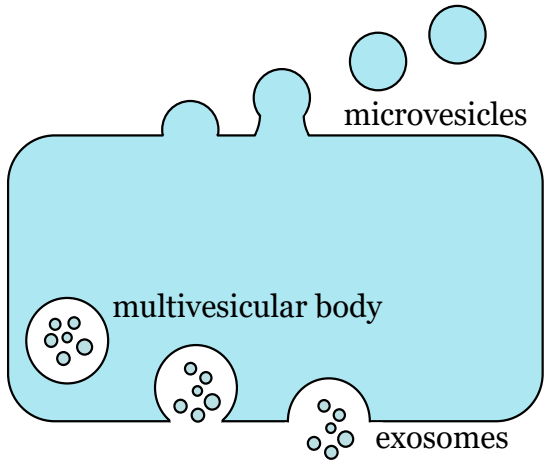

Figure 2 **Secretion of extracellular vesicles.** Exosomes are produced through the endosomal pathway while microvesicles are shed from the cell surface.

2.1.1.2 Mesenchymal stromal cells and adaptive immunity

MSCs have a plethora of effects on the immune cells of the adaptive immunity, which are summarised in **Figure 1**. T lymphocytes are essential in the formation of the adaptive immune response. They are classified into CD4+ T helper (Th) cells, CD8+ cytotoxic T cells and tolerogenic regulatory T cells (Tregs). These classes are further divided into several subpopulations. The immunosuppressive capacity of MSCs was first described in 2002, when MSCs

were reported to inhibit T-cell proliferation (Bartholomew et al. 2002; Di Nicola et al. 2002). Overall, MSCs can inhibit the proliferation and cytokine production of the CD4+ Th and CD8+ cytotoxic T cells and regulate the balance of Th1/Th2/Th17/Treg cells (González et al. 2009; Kong et al. 2009; Song et al. 2020). Moreover, MSCs can promote Treg induction (Selmani et al. 2008). Tregs modulate other effector T cells and downregulate their induction and proliferation, which improves immune tolerance and prevents the development of autoimmune diseases. Additionally, MSC-EVs are capable of affecting T-cell function, indicating that EVs at least partly mediate the effects of MSCs listed above. MSC-EVs suppress the activation of T cells and promote the apoptosis of activated T cells, alter the balance of different T-cell populations by promoting the conversion of Th1 cells to Th2 cells, suppressing the formation Th17 cells and inducing the formation of Tregs (Mokarizadeh et al. 2012; Zhang et al. 2014; Fattore et al. 2015; Chen et al. 2016; Shigemoto-Kuroda et al. 2017; Ko et al. 2020).

B cells maintain the humoral immunity by secreting antibodies. MSCs inhibit B-cell proliferation and differentiation into antibody-producing cells (Corcione et al. 2006). Moreover, MSCs can promote the survival of quiescent B cells and the differentiation of IL-10-producing CD19+CD24^{high}CD38^{high} regulatory B cells, while limiting the formation of antibody-producing plasma cells (Franquesa et al. 2015). Mediating the effects of the MSCs, MSC-EVs inhibit the proliferation, differentiation and antibody secretion of B cells (Budoni et al. 2013; Conforti et al. 2014; Di Trapani et al. 2016). Moreover, the effect can be even further enhanced with IFN-γ- and TNF-α-primed EVs (Di Trapani et al. 2016).

2.1.1.3 Mesenchymal stromal cells and innate immunity

MSCs have wide-spread effects on the different cell types of the innate immunity that provide the first line of defence against pathogens (see **Figure 1**). Neutrophils are the most abundant white blood cells and they travel in the circulation until encountering and phagocytosing a pathogen. MSCs can affect these plentiful immune cells by suppressing their respiratory burst, apoptosis and peroxidase and protease release (Jiang et al. 2016). MSC-EVs are also able to reduce the respiratory burst and infiltration of neutrophils in hepatic ischemia-reperfusion injury and ischemic brain injury models (Yao et al. 2019; Wang et al. 2020). NK cells are circulating cytotoxic lymphocytes that kill virus-infected, stressed and malignant cells. MSCs can inhibit the proliferation, cytokine production and cytotoxicity of NK cells (Aggarwal and Pittenger 2005; Poggi et al. 2005; Spaggiari et al. 2008). Interestingly, MSC-EVs are also able to suppress the proliferation of NK cells (Di Trapani et al. 2016).

Antigen-presenting dendritic cells, which bridge the adaptive and innate immunity, are modulated by MSCs, which suppress their migration, maturation and antigen presentation (Nauta et al. 2006; Chen et al. 2007;

Djouad et al. 2007; English et al. 2008). Moreover, MSCs can promote the differentiation of tolerogenic dendritic cells, which improve the immune tolerance mainly by producing anti-inflammatory cytokines and inducing Treg development (Ge et al. 2009, 2010; Spaggiari et al. 2009). The uptake of antigens and the maturation of dendritic cells were also reduced by MSC-EVs (Reis et al. 2018).

Macrophages possess a spectrum of phenotypes that have a vital impact on homeostasis, tissue repair and immunity. Tissue resident macrophages may have a prenatal origin or can differentiate from circulating peripheral blood monocytes (Ginhoux and Jung 2014). The future phenotype of monocytes arriving at the site of inflammation is determined by the inflammatory status of their microenvironment. Macrophages have high plasticity and alter their phenotypes dynamically according to the microenvironmental cues (Mosser and Edwards 2008). This plasticity can result in a mixed or transient phenotype inside the macrophage population. Traditionally, macrophages are categorised into classically activated M1 and alternatively activated M2 types, but a strict categorisation of these dynamic cells is an oversimplification. Another classification has been proposed by Mosser and Edwards (2008) with three main classes: 1) proinflammatory, host-defence macrophages, 2) wound-healing macrophages and 3) regulatory macrophages (Mregs). These classes consist of plastic and versatile populations that can gradually change into other classes. Reports also suggest that macrophages investigated *in vitro* should be named after the way they are generated and activated in cell culture, e.g., macrophages activated with IFN-γ or lipopolysaccharide (LPS) should be described as M(IFN-γ) or M(LPS), respectively (Murray et al. 2014).

Mregs represent a tolerance-promoting macrophage population with a potent T-cell suppressor function (Broichhausen et al. 2012). These macrophages produce immunosuppressive and anti-inflammatory cytokines, such as IL-10 and TGF-β1, potently suppress T-cell function and promote Treg phenotype (Hutchinson et al. 2011; Riquelme et al. 2017). Like MSCs, Mregs suppress T-cell proliferation partly via IDO signaling (Hutchinson et al. 2011). The intriguing ability of Mregs to induce tolerogenic changes has evoked interest in investigating Mregs as an adjunct therapy in renal transplants, which has proceeded into an early clinical trial (https://clinicaltrials.gov: NCT02085629).

The effects of MSCs on macrophages have received increasing attention since the late 2010s, when it was observed that MSCs can polarise macrophages towards an anti-inflammatory phenotype (Kim and Hematti 2009; Németh et al. 2009). The MSC immunomodulation has been investigated mainly in the context of resting, M1 and M2 macrophages, while the effect on Mregs remains unknown. MSCs modulate macrophages by increasing the expression of multiple cell surface markers, such as CD206, altering the cytokine production and enhancing their phagocytosis activity (Kim and Hematti 2009; Németh et al. 2009; François et al. 2012; Abumaree et al. 2013). Moreover, MSC-derived EVs have also been reported to suppress

macrophage activation, increase CD206 expression and promote macrophage polarisation towards an anti-inflammatory phenotype (Henao Agudelo et al. 2017; Lo Sicco et al. 2017; Morrison et al. 2017; Zhao et al. 2018).

2.1.2 CLINICAL USE

In June 2020, MSCs were investigated in 339 recruiting or active clinical trials worldwide (https://clinicaltrials.gov) indicating that MSCs remain to this day an attractive option for cell therapy applications. MSCs were first used as an immunomodulatory therapeutic agent in the treatment of Graft-versus-host disease (GvHD) with promising results (Le Blanc et al. 2004, 2008). Later, MSCs have been investigated in phase III clinical trials in the treatment of e.g., GvHD (Fisher et al. 2019; Kurtzberg et al. 2020), Crohn's disease (Panés et al. 2016) and chronic heart failure (Bartunek et al. 2013, 2017). The success of these clinical trials varies from negligible effects to highly promising outcomes, indicating that there are multiple aspects that need to be further improved, including determination of the dosing, fitness and immunological potency of clinical MSCs (Galipeau and Sensébé 2018). Yet, new indications for MSC therapy are still considered and MSCs have recently been investigated as an option for the treatment of coronavirus-induced disease 2019 (COVID-19) (Moll et al. 2020). Due to their regenerative properties, MSCs have also been utilised to promote e.g., the bone regeneration of mandibular fractures (Castillo-Cardiel et al. 2017) and cartilage repair in osteoarthritis (Zhang et al. 2019).

To date, MSCs have been administered in numerous clinical trials and the therapy has been deemed safe (Galipeau and Sensébé 2018). One of the advantages of MSCs is that they do not generally engraft and are cleared from the body relatively quickly, reducing the risk of oncogenicity (von Bahr et al. 2012a). However, the clearing of MSCs has puzzled researchers and the determination of complete immunomodulatory mechanisms of action has been a challenge. This has led to the idea of hit-and-run mechanism, according to which MSCs induce their therapeutic effect by secreting paracrine molecules and EVs and subsequently modulating immune cells, before being cleared from the body (von Bahr et al. 2012a; Prockop 2017).

Because EVs can mediate the MSC therapeutic response, MSC-EVs have been investigated as an option for the traditional MSC therapy in *in vivo* models (Bruno et al. 2009; Lai et al. 2010) and patients with encouraging results (Kordelas et al. 2014; Nassar et al. 2016). MSC-EVs have elicited beneficial effects in several *in vivo* models, such as acute kidney injury (Bruno et al. 2009; Kilpinen et al. 2013a), acute myocardial infarction (Lai et al. 2010) and ischemic stroke (Xin et al. 2013; Doeppner et al. 2015). Moreover, MSC-EVs were reported to have improved the condition of a patient with acute steroid-resistant GvHD (Kordelas et al. 2014). MSC-EVs were also observed to improve the symptoms and cytokine profile of patients with chronic kidney disease in a phase II/III clinical pilot study (Nassar et al. 2016). Other clinical

trials employing MSC-EVs have been registered, but the results have not yet been reported. In June 2020, MSC-EVs were investigated in two recruiting-phase clinical trials (https://clinicaltrials.gov). Importantly, the administration of MSC-EVs has not resulted in adverse side effects *in vivo* or in patients even after a one-year follow-up, indicating the safety of MSC-EV therapy (Kordelas et al. 2014; Nassar et al. 2016; Börger et al. 2017).

Overall, the cell-free EV therapy has advantages over the normal cell therapy (Lener et al. 2015; Giebel et al. 2017). For example, EV fractions can be filtrated to remove pathogens and EVs cannot self-replicate reducing the risk of oncogenic side-effects. Both of these factors increase the safety of the administered therapeutics. Additionally, MSCs are more susceptible to experiencing an altered phenotype or loss of potency than EVs due to storage conditions, such as freezing and thawing. The research of MSC-EVs is thus likely to continue further into the clinics.

2.2 RESOLUTION OF INFLAMMATION

Inflammation is a dynamic process, which can be divided into distinct phases. The main two phases are the acute and resolution phases, the simplified characteristics of which are depicted in **Figure 3**. Many factors, such as cytokines, chemokines and, most importantly, LMs, regulate these phases.

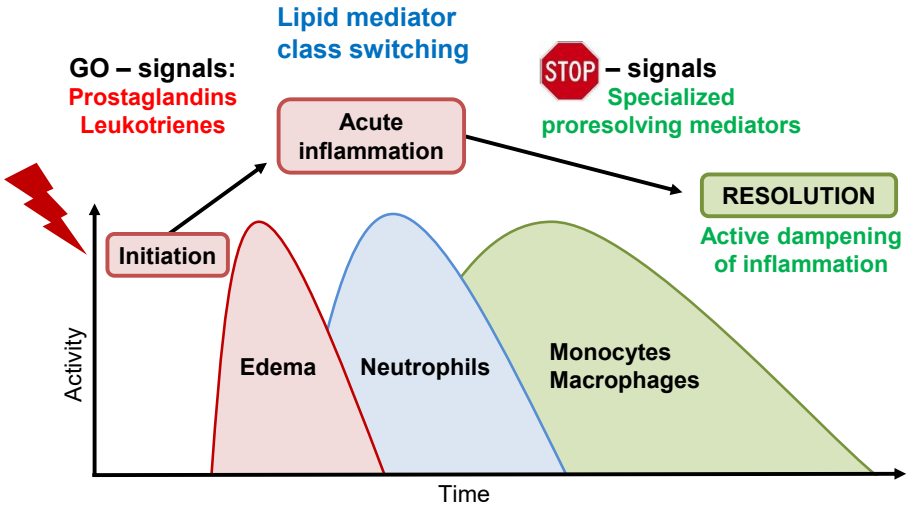

Figure 3 **Schematic overview of the acute and resolution phase of inflammation.** Model adapted from Serhan and Petasis (2011).

The acute inflammatory phase is the first, and crucial, phase in the defence system against pathogens and tissue injury. The acute inflammatory response is characterised by five cardinal signs: 1) fever, 2) erythema (redness), 3) oedema, 4) pain and 5) loss of function, which are dependent on the severity of the inflammatory state (Basil and Levy 2016). The major mediators of this acute inflammatory response are LMs: PGs and leukotrienes (LTs), which induce vascular permeability and neutrophil chemotaxis, for example. Proinflammatory cytokines and chemokines have overlapping effects with the proinflammatory LMs.

Previously, it was believed that acute inflammation would passively dampen with the dilution of proinflammatory molecule gradients. However, it is currently well established that the dampening of inflammation is actively orchestrated and regulated. This second phase, the resolution of inflammation, has also been assigned five cardinal signs consisting of 1) removal of microbes, dead cells and debris, 2) restoration of vascular integrity and perfusion, 3) regeneration of tissue, 4) remission of fever and 5) relief of pain (Basil and Levy 2016). Resolution is regulated by a variety of factors such as protein annexin A1, chemerin-derived peptides, neurotransmitter acetylcholine and, most importantly, specialized proresolving mediators (SPMs) (Headland and Norling 2015). SPMs are novel LMs that are derived from n-3 PUFAs eicosapentaenoic acid (EPA), n-3 docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA), and also from n-6 PUFA AA (Serhan 2017). SPMs are classified into resolvins, protectins, maresins and lipoxins, and they elicit essential functions in regulating resolution at pico- to nanomolar concentrations (described in more detail in **Section 2.3.3**). The main effects of the proresolving agents are the cessation of neutrophil recruitment to the inflammatory site, the polarisation of macrophages towards an anti-inflammatory and proresolving phenotype and increased efferocytosis by macrophages.

In addition, a third phase, post-resolution, has been proposed to follow the resolution of inflammation (Motwani et al. 2017; Newson et al. 2017). During the post-resolution phase, sustained PGE_2 production suppresses the innate immunity and predisposes the tissue to bacterial infection. At the same time, the PGE_2 production hinders the tendency to develop autoimmune responses, indicating that the post-resolution has an important safety-related function in the returning of the homeostasis.

A dysregulated and uncontrolled acute inflammation may lead to the formation of a chronic inflammation, which causes tissue damage and the formation of various diseases. A failure to resolve an acute inflammation underlies behind numerous inflammatory diseases common in the Western countries, such as cardiovascular diseases (Libby et al. 2002), Crohn's disease (Feuerstein and Cheifetz 2017), rheumatoid arthritis (Choy and Panayi 2001) and Alzheimer's disease (Koistinaho and Koistinaho 2005). Moreover, the dysregulation of SPMs has been linked to the pathogenesis of various diseases,

highlighting the importance of these proresolving LMs in the dampening of inflammation (Basil and Levy 2016).

2.3 LIPIDS, FATTY ACIDS AND LIPID MEDIATORS

Lipids have vital roles in the physiology of cells. Lipids serve as an energy storage and form cell membranes, which are essential for cell function (van Meer et al. 2008). Furthermore, it has been established that membrane phospholipids, fatty acids attached to them as acyl chains and especially fatty acid-derived LMs have multiple roles in cell metabolism, signalling and inflammation (Serhan et al. 2015; Sunshine and Iruela-Arispe 2017).

Lipids are a diverse group of molecules and have been classified into eight categories comprising fatty acyls, glycerolipids, glycerophospholipids (GPs), sphingolipids, sterol lipids, prenol lipids, saccharolipids, polyketides (Fahy et al. 2005). The lipid categories investigated in this thesis are fatty acyls, GPs and sphingolipids. The fatty acyl category contains fatty acids and their enzymatically biosynthesised derivatives LMs. GPs and sphingolipids are membrane phospholipids, which consists of several subclasses. A simplified diagram of membrane phospholipid asymmetry, fatty acid incorporation and LM biosynthesis is presented in **Figure 4**.

Lipids and their derivatives are commonly profiled with mass spectrometry-based lipidomics employing soft ionisation techniques, such as electrospray ionisation-tandem mass spectrometry (ESI-MS/MS) or matrix-assisted laser desorption ionisation (Wenk 2010). Due to the diverse chemical properties of lipids, it is impossible to profile all lipid classes from the same sample because of the different extraction and analysis methods. Typical analysis methods employ mass spectrometry with direct infusion of the sample solution to the ion source or combine the mass spectrometer on-line with liquid chromatography or gas chromatography (GC), which pre-separate different lipids prior to the detection.

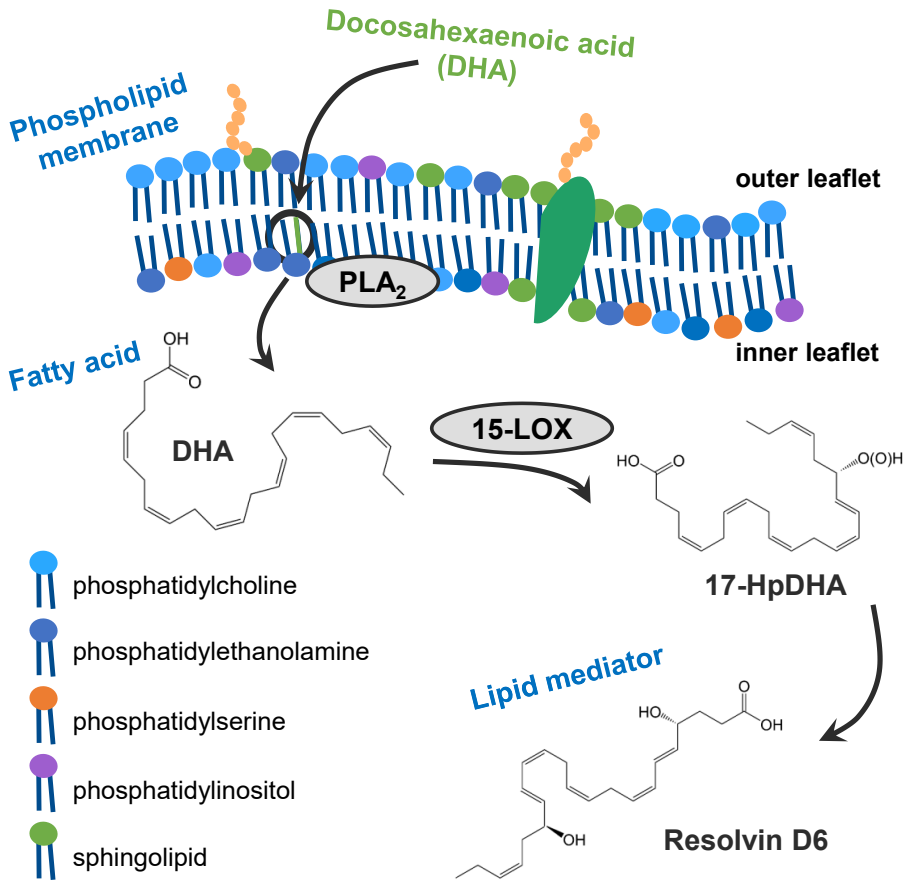

Figure 4 **A simplified diagram on plasma membrane phospholipids (e.g., cholesterol molecules not shown), fatty acid incorporation and lipid mediator biosynthesis.** PLA_2, phospholipase A_2; 15-LOX, 15-lipoxygenase; 17-HpDHA, 17-hydroperoxydocosahexaenoic acid.

2.3.1 PHOSPHOLIPIDS

Phospholipids are amphipathic membrane lipids that form the plasma and organelle membrane bilayers (Fahy et al. 2005; van Meer et al. 2008). The polar head and nonpolar acyl chains give rise to the bilayer structure of membranes, where the polar heads face outwards towards the water solution and the hydrophobic tails face each other. The diversity of phospholipids arises from different combinations of head groups and especially of the fatty acyl chains, resulting in more than 1,000 possible phospholipid species in eukaryotic membranes. The phospholipid composition of the outer and inner leaflets of the plasma membrane is asymmetrical. This asymmetry is maintained with flippases, also known as aminophospholipid translocases, that transport amine-containing phospholipids from the outer to inner leaflet

(Segawa and Nagata 2015). Flippases counteract the activity of scramblase and floppase enzymes. Scramblases transport lipids bidirectionally into both outer and inner leaflet, and floppases transport lipids from the inner leaflet to the outer one.

GPs and sphingolipids are phospholipids that have different structural properties (van Meer et al. 2008). GPs contain a glycerol that connects the phosphoric acid-containing head group and variable fatty acyl chains; typically, a saturated fatty acyl is located in the *sn*-1 position and an unsaturated fatty acyl in the *sn*-2 position. LysoGPs have only one acyl chain attached to the polar head, while the other position holds only a hydroxyl group. Sphingolipids consists of a sphingoid backbone (e.g., a sphingosine) that is amide-linked to one fatty acyl chain. In mammals, these fatty acyls are mainly saturated or monounsaturated rather than di- or polyunsaturated fatty acyls. Like GPs, sphingolipids have different head groups attached to the sphingoid backbone.

GPs and sphingolipids are further classified into subclasses based on their head groups (Fahy et al. 2005; van Meer et al. 2008). Phosphatidylcholines (PCs) contain a choline head group, and form the largest phospholipid class in eukaryotic cells, contributing over 50% of most membrane lipids. PCs are mainly found in the outer leaflet, while phosphatidylethanolamines (PEs), 20% of membrane lipids, are mainly present in the inner leaflet. PEs have a relatively small ethanolamine head group, which gives rise to the conical shape of the molecule. When transported in excess to the outer leaflet, this shape causes stress to the bilayer and contributes to microvesicle formation and budding (Wehman et al. 2011; Beer et al. 2018).

Phosphatidylserine (PS) class, with serine head group, is also typically present in the inner leaflet of the plasma membrane (van Meer et al. 2008). In apoptotic cells, PS is transported to the cell surface, which serves as a signal to phagocytic cells to engulf these apoptotic cells (Segawa and Nagata 2015). Both PEs and PSs are amine-containing phospholipids and their asymmetry is established with flippases.

One infrequent, but crucial, membrane GP class is phosphatidylinositols (PIs), which have an important role in cell signalling as the precursor for important second messengers inositol 1,4,5,-triphosphate and diacylglycerol (van Meer et al. 2008; Sunshine and Iruela-Arispe 2017). Other GP groups include phosphatidic acid, phosphatidylglycerol and cardiolipins, which are relatively infrequent on membranes.

The sphingolipid class ceramides only have a hydroxyl group as a head group, while other sphingolipid classes contain larger head groups. For example, sphingomyelins (SMs) contain a choline head group and glycosphingolipids have carbohydrate-containing head groups. SMs and glycosphingolipids are mainly located on the outer leaflet of plasma membrane.

2.3.2 FATTY ACIDS

Hydrophobic fatty acids are present in cells mainly as acyl chains of triacylglycerols and sterol esters in lipid droplets or membrane phospholipids, and they are also found in cytosol bound to carrier proteins (Ratnayake and Galli 2009). Fatty acids consist of an aliphatic carbon chain with a carboxyl group at one end. Fatty acids can be categorised in multiple ways, some of which are presented here. Most commonly, they are categorised on the basis of the length and degree of saturation, i.e., the number of double bonds, of the carbon chain. Saturated fatty acids do not contain any double bonds and may be further categorised according to the length of their aliphatic carbon chain. Unsaturated fatty acids, on the other hand, have at least one double bond between two carbons in the aliphatic chain. These fatty acids are further classified into monounsaturated fatty acids (MUFAs, one double bond) and PUFAs (≥ two double bonds). The main naturally occurring double bond configuration is *cis*, which causes a bend in the fatty acid. Thus, the fluidity of cellular membranes increases with the increasing degree of unsaturation of the phospholipid acyl chains. The uncommon *trans* configuration of a double bond leaves the carbon chain straight rather than bent. *Trans* fatty acids are to some extent more common in ruminants than other mammals and can also arise from the technological treatment of fats (Sommerfeld 1983).

There are different ways to name fatty acids: the n-, ω- and Δ-nomenclature, and fatty acids also have trivial names (Cook and McMaster 2002; Ratnayake and Galli 2009). In this thesis, the trivial names and the n-nomenclature system are used. In n-nomenclature, fatty acids are named based on their chemical structure. For example, DHA (22:6n-3) has 22 carbons, 6 double bonds, and the first double bond is located in the third carbon calculated from the terminal methyl carbon, which is called ω-position. There are multiple PUFA families based on the first double bond in the n-position (from n-1 to n-12), but the most common naturally occurring families are n-3, n-6, n-7 and n-9 (Cook and McMaster 2002).

Fatty acids are biosynthesised *de novo* with the fatty acid synthase enzymatic system in the cytosol (Cook and McMaster 2002). Fatty acids generated *de novo* or obtained through nutrition can be modified with desaturases (addition of a double bond), elongases (lengthening the carbon chain by two carbons) in endoplasmic reticulum and partial β-oxidation (shortening of fatty acids) in peroxisomes. Because mammalian cells lack certain desaturases and, thus, cannot synthesise n-3 and n-6 fatty acid family precursors, these fatty acids must be acquired from dietary sources. The essential fatty acids are linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3), which are converted with methyl-end Δ12- and Δ15-desaturases, respectively, and synthesised in phytoplankton, plants, lower eukaryotes and certain animals, such as nematodes, cockroaches and house crickets (Cripps et al. 1990; Okuley et al. 1994; Sakuradani et al. 1999; Cook and McMaster 2002; Zhou et al. 2011).

With front-end desaturases, elongases and partial β-oxidation in peroxisomes, eukaryotic cells can process fatty acids into longer-chained PUFAs. However, the different n-families compete for these same enzymes (Cook and McMaster 2002). The n-3 fatty acids are better substrates for the Δ6 desaturase than n-6, while n-6 fatty acids are favoured over n-9 family precursors. In a state of LA and ALA deficiency, animal cells begin desaturating and elongating 18:1n-9 fatty acid to produce 20:3n-9. This fatty acid can compensate for the PUFA requirements in the membrane fluidity but is not as beneficial from a physiological perspective because it is not a precursor to pivotal LMs. LA and ALA deficiency is uncommon in people following the Western diet, but it is often observed in cell culture, where the fatty acids are obtained from cell culture medium and the PUFA content may be suboptimal (Karmiol and Bettger 1988; Kiamehr et al. 2018).

The n-6 precursor PUFA LA is found in high levels in sunflower, safflower, soybean and corn oils and is abundantly obtained from the Western diet (Patterson et al. 2012). LA can be desaturated and elongated to AA (20:4n-6), which is vital to all cells from a structural, physiological and inflammatory perspective (Cook and McMaster 2002; Ratnayake and Galli 2009; Stables and Gilroy 2011). With an appropriate stimulus, AA is readily deliberated from membrane phospholipids by especially phospholipase A_2 (PLA_2) and is then converted into mainly proinflammatory LMs inside the cell (Stables and Gilroy 2011). AA can also be obtained from terrestrial animal fats and eggs (Ratnayake and Galli 2009). Although these fatty acids are essential and important for the normal functions of the body, the intake of n-6 PUFAs in the Western diet is not limited but rather excessive compared with n-3 PUFA intake (Ratnayake and Galli 2009; Patterson et al. 2012). This excess consumption of n-6 PUFAs most likely contributes to the increased incidence of cardiovascular and other chronic inflammatory diseases in the industrialised countries (Patterson et al. 2012).

The n-3 precursor PUFA ALA is primarily present in plant oils, especially flaxseed and perilla oils (Ratnayake and Galli 2009). Eukaryotes can process ALA into EPA (20:5n-3), n-3 docosapentaenoic acid (DPA, 22:5n-3) and DHA (22:6n-3) (Cook and McMaster 2002; Ratnayake and Galli 2009). n-3 DPA is an intermediate product in the conversion of EPA to DHA via the Sprecher pathway and is less abundant than the other two n-3 PUFAs (Sprecher and Chen 1999). EPA and DHA are originally biosynthesised in phytoplankton and due to accumulation in the food chain, the main dietary sources are fatty marine fish, such as salmon and herring (Ratnayake and Galli 2009). These two PUFAs, which regulate membrane fluidity, transcription and cell signalling, are vital for organs, such as brain and eyes, and have multiple beneficial effects on inflammatory diseases (Arterburn et al. 2006; Calder 2006, 2015). Interestingly, the incidence of inflammatory diseases is lower in countries with diets rich in of n-3 marine oils (Bang and Dyerberg 1980; Menotti et al. 1999). The beneficial effects of n-3 PUFAs have been demonstrated in *in vivo* experiments although the impact of EPA and DHA

supplements in clinical trials remains unclear (Leslie et al. 1985; Vilaseca et al. 1990; Calder 2008; Miles and Calder 2012). The anti-inflammatory mechanisms of EPA and DHA include decreased AA-derived signalling, resulting from competitive inhibition and leading to decreased production of inflammatory cytokines and leukocyte chemotaxis (Holman and Mohrhauer 1963; Calder 2006). Notably, the beneficial effects of n-3 PUFAs are most likely associated with the conversion of these PUFAs into SPMs that promote the resolution of inflammation (**discussed in more detail in Section 2.3.3**) (Calder 2006, 2015; Serhan et al. 2015).

Moreover, it is important to obtain EPA and DHA directly from the diet instead of consuming only the precursor ALA. It has been reported that the modification of ALA is limited in humans, with 0.3 to 21% of the ALA consumed converted into EPA and only 1 to 9% into DHA (Arterburn et al. 2006). Moreover, the ability to convert precursor PUFAs into long-chain PUFAs varies in different cell types, and mainly occurs in hepatocytes. These observations highlight the importance of dietary consumption of long-chain n-3 PUFAs and the need for fatty acid supplements in *in vitro* cell cultures.

2.3.3 LIPID MEDIATORS

LMs are enzymatically converted bioactive derivatives of fatty acids. These molecules have received growing attention during the past decades due to the expanding knowledge on their pivotal role in cellular processes and especially in inflammation (Stables and Gilroy 2011; Serhan et al. 2015). A more detailed description of the effects of LMs in inflammation is found in **Section 2.3.3.5**. The key PUFA precursors for LMs in inflammatory processes elucidated to date are AA, EPA, n-3 DPA and DHA. The LMs derived from these precursors are depicted in **Figure 5**.

According to the classical view, the membrane-bound PUFAs are liberated by PLA_2 and enzymatically converted into bioactive LMs (Buczynski et al. 2009; Stables and Gilroy 2011). Interestingly, LMs or their biosynthetic intermediates may also be esterified in membranes (Hammond and O'Donnell 2012). The biosynthesis of LMs involves multiple steps, including typically the formation of transient hydroperoxyl or epoxide intermediates (Serhan and Levy 2018). The hydroperoxyl intermediates are rapidly converted into more stable hydroxyl molecules that can be used as biosynthetic pathway markers. The hormone-like LMs are autocrine or paracrine molecules that affect the cell of origin or the neighbouring cells by binding to G protein-coupled receptors, which initiates multiple signalling cascades (Buczynski et al. 2009; Pirault and Bäck 2018). Thus, LMs are readily inactivated with the formation of further metabolites. A common inactivation method is ω-oxidation, where a hydroxyl or carboxyl group is attached to the ω-end of the molecule, a reaction catalysed by cytochrome P450 (CYP450) enzymes (Petasis et al. 2005; Buczynski et al. 2009). LMs may also be inactivated by dehydrogenases that form oxo-

metabolites with an hydroxyl group converted into a ketone group (Petasis et al. 2005).

A key finding related to the progress and pathophysiology of inflammation was the elucidation of AA-derived PGs. PGs were first observed in the 1930s in multiple independent reports (see review by Von Euler, 1936), but the structure and physiological actions were described in the 1950-60s by Professors Sune Bergström and Bengt Samuelsson and colleagues (Bergström and Samuelsson 1965). It was also discovered that AA was the precursor of 2-series PGs (Bergström et al. 1964; van Dorp et al. 1964). These advances led to the observation by Sir John R. Vance that aspirin-like drugs could inhibit PG synthesis (Vane 1971). Consequently, the importance of the biosynthetic enzyme cyclooxygenase (COX)-2 in inflammation was elucidated and various drugs inhibiting this enzyme have been developed. These discoveries in the field of PGs were acknowledged by a Nobel Prize in Physiology or Medicine in 1982, while the research on eicosanoids, i.e., the 20-carbon LMs, progressed further. The classical proinflammatory eicosanoids have been under intensive research due to their importance in inflammatory diseases and drug development. Nowadays, there is a growing interest in promoting disease resolution rather than focusing only on inflammation suppression (Dalli and Serhan 2019).

The elucidation of the anti-inflammatory mechanisms of n-3 PUFAs was greatly advanced by the findings of Professor Charles N. Serhan and colleagues, when they identified and determined the function of proresolving LMs, termed SPMs. SPMs consists of multiple LM classes: resolvins, protectins, maresins and lipoxins, which are derived from EPA, DHA, n-3 DPA and AA (Serhan et al. 1984, 2000, 2002, 2009; Hong et al. 2003; Dalli et al. 2013, 2014, 2015a). Moreover, Serhan and his colleagues developed and are constantly improving a highly sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based method to identify and quantify LMs. To this day, novel SPMs are discovered at a steady rate, and most likely multiple SPMs are yet to be found.

LMs and other enzymatically oxygenated molecules are also synthesised from other PUFAs, such as dihomo-γ linolenic acid (DGLA, 20:3n−6) and n-6 DPA (22:5n-6) (Bergström et al. 1964; Fischer 1989; Dangi et al. 2009). DGLA is the precursor to 1-series PGs, thromboxanes and 3-series LTs (Bergström et al. 1964; van Dorp et al. 1964; Fischer 1989). n-6 DPA is a precursor to e.g., monohydroxylated 17-hydroxydocosapentaenoic acid (HDPA)n-6 and dihydroxylated 10,17-HDPAn-6, which have been reported to elicit anti-inflammatory activities (Dangi et al. 2009; Chiu et al. 2012). Moreover, the n-3 and n-6 PUFAs ALA and LA, respectively, can be lipoxygenated into mono- and dihydroxylated molecules (Liu et al. 2013; Wennman et al. 2015). Due to the improved analytical methods and an increase in the number of the PUFA-derived oxygenated molecules identified, it is likely that new LM precursors are still waiting to be discovered.

In the following sections, LMs that have essential roles in the different phases of inflammation and their simplified biosynthetic pathways are described.

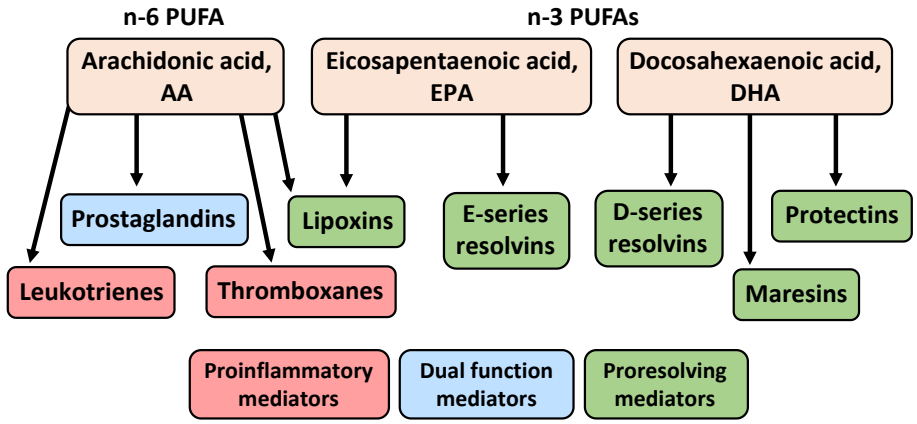

Figure 5 **A simplified diagram on the biosynthetic routes of lipid mediators derived from arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid.** PUFA, polyunsaturated fatty acid.

2.3.3.1 AA-derived lipid mediators

AA is a precursor to predominantly proinflammatory 2-series PGs and thromboxanes, 4-series LTs and proresolving 4-series lipoxins as well as several other oxygenation products such as epoxide-derivatives epoxyeicosatrienoic acids and endocannabinoids (Bergström et al. 1964; van Dorp et al. 1964; Borgeat and Samuelsson 1979; Lewis et al. 1980; Serhan et al. 1984). Even though PGs have traditionally been considered proinflammatory, especially PGE_2 and PGD_2 have been described to have more complex actions, eliciting also anti-inflammatory effects, such as in the function of MSC immunomodulation.

PG and thromboxane biosynthesis from AA is catalysed by PGH synthase-1 and 2, also known as COX-1 and 2, which are bifunctional enzymes containing both peroxidase and cyclooxygenase activities (Stables and Gilroy 2011). These enzymes are typically referred only as COX enzymes. In a simplified view, COX-1 is constitutively expressed in most cells, while COX-2 is an inducible enzyme that is rapidly expressed after an inflammatory stimulus (Dubois et al. 1998). After the COX-mediated conversion, the consequent PGH_2 intermediate is further converted into PGD_2, PGE_2, PGI_2, $PGF_{2\alpha}$ and thromboxane A_2 (TXA_2), each with their own synthases (Stables and Gilroy 2011). In general, these LMs are produced in most tissues and cell types, but the expression rate of these synthases determines the final LM profile. TXA_2 is a very short-lived molecule with a half-life of only ~30 seconds and is rapidly converted into a more stable thromboxane B_2 (TXB_2)

(Granström et al. 1976). PGs elicit their actions by binding to EP1-4 receptors and TXA_2 to TP receptor (Stables and Gilroy 2011).

LTs are mainly biosynthesised in immune cells, such as neutrophils and macrophages. LTs consist of LTB_4 and its metabolome, and cysteinyl LTs LTC_4, LTD_4 and LTE_4, the which are peptidoconjugates (Borgeat and Samuelsson 1979; Lewis et al. 1980). The conversion of AA is catalysed by 5-lipoxygenase (LOX), which oxidises AA into 5-hydroperoxyeicosatetraenoic acid (HpETE) with the support of 5-LOX activating protein FLAP. With the removal of water, HpETE is converted into an unstable epoxide LTA_4, which is either hydroxylated into LTB_4 or converted into LTC_4 with the addition of a glutathionyl group. With the removal of glutamic acid, LTC_4 is converted into LTD_4 and a subsequent removal of glycine yields LTE_4. Cysteinyl LTs bind to cysteinyl leukotriene receptor (CysLT)1 and CLT2, while LTB_4 binds to B leukotriene receptor (BLT)1 and BLT2.

Proresolving lipoxins were first described in 1984 in neutrophils (Serhan et al. 1984). The identified lipoxins include lipoxin A_4 (LXA_4), 15-epi-LXA_4, lipoxin B_4 (LXB_4) and 15-epi-LXB_4 (Serhan et al. 1984). Lipoxin synthesis can occur via three pathways. 15-LOX catalyses the formation of 15-HpETE, which is further converted by 5-LOX and lipoxin hydrolases into LXA_4 and LXB_4. The epi-lipoxins are synthesised in a similar manner, but the first oxygenation is catalysed by aspirin-acetylated COX-2 or CYP450 enzymes to produce lipoxins with *R* stereochemistry (Clària et al. 1996). It is good to bear in mind that the ratio of the produced *R*- and *S*-epimers varies in different CYP450 enzyme isoforms (Westphal et al. 2011). With a joint operation of leukocytes and platelets, LTA_4 can be converted with neutrophil-derived 5-LOX and platelet-derived 12-LOX into LXA_4 and LXB_4. Lipoxins are known to bind ALX/FPR2 and CysLT1 receptors (Gronert et al. 1998; Krishnamoorthy et al. 2010).

2.3.3.2 EPA-derived lipid mediators

EPA is the precursor to 3-series PGs and thromboxanes, and 5-series LTs and lipoxins. These LMs are synthesised in a similar manner to their counterparts derived from AA; however, the function of these EPA-derived LMs is different (Schmitz and Ecker 2008). For example, the 3-series PGs and 5-series LTs are more anti-inflammatory and less potent than their AA-derived counterparts. More importantly, Serhan and colleagues discovered in 2000 that EPA is also a precursor to a novel class of proresolving LMs (Serhan et al. 2000). To date, E-series resolvins (RvEs) consisting of 18-hydroxyeicosapentaenoic acid (HEPE), RvE1, RvE2 and RvE3 (both *R* and *S* epimers) have been identified and their actions elucidated (Serhan et al. 2000; Arita et al. 2005; Tjonahen et al. 2006; Oh et al. 2011; Isobe et al. 2012, 2013).

The synthesis of RvEs is initiated with the formation of 18*R*- or 18*S*-HEPE by CYP450 or aspirin-acetylated COX-2 (Hong et al. 2003; Arita et al. 2005). 18*R*- or 18*S*-HEPE are oxygenated by 5-LOX in neutrophils and the intermediate is hydrolysed by LTA_4 hydrolase to yield RvE1 and 18*S*-RvE1

(Serhan et al. 2000; Arita et al. 2005; Oh et al. 2011) or reduced to yield RvE2 and 18*S*-RvE2 (Tjonahen et al. 2006). RvE3 can also be synthesized from 18-HEPE via lipoxygenation by 15-LOX in eosinophils or resident macrophages (Isobe et al. 2012, 2013). The term RvE3 covers both *R*- and *S*-diastereomers of the molecule. The effects of RvE1 and RvE2 are mediated by ERV1/ChemR23 receptor, but these molecules also bind as antagonists to BLT1, blocking LTB_4 mediated signalling (Arita et al. 2005, 2007; Oh et al. 2012). Interestingly, the monohydroxy 18-HEPE is also bioactive and cardioprotective (Endo et al. 2014).

2.3.3.3 DHA-derived lipid mediators

The DHA-derived SPMs were first discovered in 2002, when Serhan and colleagues discovered the D-series resolvins (RvDs) and elucidated the importance of LOXs and aspirin-acetylated COX-2 in the formation of these novel SPMs (Serhan et al. 2002; Hong et al. 2003). In the coming years, DHA-derived protectins (Hong et al. 2003; Marcheselli et al. 2003; Mukherjee et al. 2004; Chen et al. 2009) and maresins (Serhan et al. 2009) were also discovered.

The biosynthesis of the 17*S*-epimer RvDs (RvD1, RvD2, RvD3, RvD4, RvD5 and RvD6) is initiated by 15-LOX, which catalyses the lipoxygenation of DHA into 17*S*-hydroperoxydocosahexaenoic acid (HpDHA) (Serhan et al. 2002; Hong et al. 2003; Serhan and Petasis 2011). With a second lipoxygenation at C-7 position, 17*S*-HpDHA is converted into a peroxide intermediate followed by an epoxide intermediate. RvD1 and RvD2 are synthesised with the enzymatic hydrolysis of the epoxide and RvD5 through the reduction of the peroxide intermediate. With 5-LOX lipoxygenation at C-4 position of 17*S*-HpDHA, RvD3, RvD4 and RvD6 are formed in a similar manner as RvD1, RvD2 and RvD5, respectively. The formation of 17*R*-mediators (17*R*-RvD1-RvD6) occurs similarly to the 17*S*-mediators, with the difference that DHA is initially converted into 17*R*-HpDHA by aspirin-acetylated COX-2 or CYP450 enzymes.

Protectins are biosynthesised from the same 17-HpDHA intermediate as RvDs (Hong et al. 2003; Marcheselli et al. 2003; Mukherjee et al. 2004; Chen et al. 2009). Protectin D1 (PD1, also known as neuroprotectin D1), is formed through enzymatic epoxidation and hydrolysis of 17*S*-HpDHA, while protectin DX (PDX, also known as 10*S*,17*S*-diHDHA) is formed though double lipoxygenation. 17*R*-epimer protectin biosynthesis is initiated with the formation of 17*R*-HpDHA by aspirin-acetylated COX-2 or CYP450 enzymes.

In contrast to RvDs and protectins, maresin biosynthesis is initiated with the lipoxygenation catalysed by 12-LOX with the conversion of DHA into 14*S*-HpDHA (Serhan et al. 2009; Deng et al. 2014). This intermediate molecule goes through enzymatic epoxidation and hydrolysis by a hydrolase to yield maresin 1 (MaR1) or soluble epoxide hydrolase to yield maresin 2 (MaR2). As with cysteinyl LTs, the peptidoconjugates of resolvins, protectins and

maresins have been identified (Dalli et al. 2014, 2015b). These molecules are referred to as resolvin, protectin and maresin conjugates in tissue regeneration (RCTR, PCTR and MCTR, respectively), with the same three corresponding peptidoconjugates as for cysteinyl LTs.

The receptors mediating the effects of RvDs include ALX/FPR2, DRV1/GPR32 and DRV2/GPR18 (Krishnamoorthy et al. 2010, 2012; Chiang et al. 2015). Recently, it was discovered that MaR1 binds to LGR6 receptor found in phagocytes, while other receptors for maresins and protectins remain to be elucidated (Chiang et al. 2019).

2.3.3.4 n-3 DPA-derived lipid mediators

An intermediate fatty acid in the conversion of EPA to DHA, n-3 DPA, is also a precursor to n-3 DPA-derived D-series resolvins ($RvD_{n\text{-}3\ DPA}$), protectins ($PD_{n\text{-}3\ DPA}$), maresins ($MaR_{n\text{-}3\ DPA}$) and 13-series resolvins (RvTs) (Dalli et al. 2013, 2015a). RvT is the most recent SPM family elucidated by Dalli et al. (2015) when RvT1, RvT2, RvT3 and RvT4 were discovered. The biosynthesis of RvTs has been partly elucidated with the COX-2-mediated oxygenation of n-3 DPA into 13*R*-HDPA (Dalli et al. 2015a; Primdahl et al. 2016). The formation of RvTs most likely occurs via 5-LOX lipoxygenation in neutrophils. Intriguingly, similarly to aspirin-acetylated COX-2, statins can increase the formation of *R*-epimer SPMs, such as RvTs, via the *S*-nitrosylation of COX-2 (Dalli et al. 2015a).

The biosynthesis of $RvD_{n\text{-}3\ DPA}$, $PD_{n\text{-}3\ DPA}$ and $MaR_{n\text{-}3\ DPA}$ series mediators occurs in a similar manner to the biosynthesis of their DHA-counterparts. The known $RvD_{n\text{-}3\ DPA}$ family currently comprises $RvD1_{n\text{-}3\ DPA}$, $RvD2_{n\text{-}3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$, and the $PD_{n\text{-}3\ DPA}$ family of $PD1_{n\text{-}3\ DPA}$ and $PD2_{n\text{-}3\ DPA}$. These mediators are biosynthesised from n-3 DPA through lipoxygenation by 15-LOX to form 17*S*-hydroperoxydocosapentaenoic acid (HpDPA), which is further lipoxygenated with 5-LOX to yield $RvDs_{n\text{-}3\ DPA}$ (Dalli et al. 2013). To form $PD_{n\text{-}3\ DPA}$, 17*S*-HpDPA is converted into an epoxide intermediate, which is further converted into $PD1_{n\text{-}3\ DPA}$ and $PD2_{n\text{-}3\ DPA}$ by epoxide hydrolases (Dalli et al. 2013; Primdahl et al. 2016; Pistorius et al. 2018). The $MaR_{n\text{-}3\ DPA}$ family includes $MaR1_{n\text{-}3\ DPA}$, $MaR2_{n\text{-}3\ DPA}$ and $MaR3_{n\text{-}3\ DPA}$, which are biosynthesised from n-3 DPA via 12-LOX lipoxygenation to form 14*S*-HpDPA. This molecule is converted into an epoxide intermediate, which is hydrolysed to yield $MaR1_{n\text{-}3\ DPA}$ and $MaR2_{n\text{-}3\ DPA}$, while the biosynthesis of $Mar3_{n\text{-}3\ DPA}$ remains to be elucidated (Dalli et al. 2013). Recently, it was shown that $RvD5_{n\text{-}3\ DPA}$ mediates its protective effects via GPR101 receptor (Flak et al. 2020).

2.3.3.5 Lipid mediators in resolution of inflammation

The proinflammatory PGs, LTs and thromboxanes participate in the initiation of the acute inflammatory response. PGs induce vasodilatation and sensitivity

to pain whereas LTs and thromboxanes induce vasoconstriction and vascular permeability (Serhan 2017). Additionally, LTB_4 is a powerful chemoattractant, which promotes the migration of neutrophils to the inflammatory site (Petri and Sanz 2018). Although PGs have received attention primarily as proinflammatory molecules, PGD_2, PGE_2 and PGI_2 also have anti-inflammatory and immunosuppressive effects (Weinberg and Weston 1985; Takayama et al. 2002; Gilroy et al. 2004). As described in earlier sections, PGE_2 is one of the main mediators of the MSC immunomodulatory response initiating anti-inflammatory effects.

Interestingly, PGE_2 and PGD_2 are also involved in the LM class switching (Levy et al. 2001). As part of this phenomenon, the production of proinflammatory LMs is decreased and the production of SPMs is increased. During acute inflammatory phase, PGE_2 can inhibit the production of LTB_4 possibly through 5-LOX inactivation and induce lipoxin production though 15-LOX activation. The induction of the 15-LOX gene transcription is partly mediated by elevated cAMP levels. Thus, the acute inflammatory response is required to initiate the production of SPMs and to reach resolution and homeostasis.

Different SPMs have been assigned various, and often overlapping, functions. This is partly due to the fact that many SPMs are agonists for the same G protein-coupled receptors (Chiang and Serhan 2017). Interestingly, the vagus nerve controls resolution and can stimulate SPM production (Mirakaj et al. 2014; Serhan et al. 2018). SPMs potently regulate the functioning of the innate immune system, especially of neutrophils and macrophages. All classes of SPMs have the ability to limit neutrophil recruitment to the inflammatory site (Headland and Norling 2015). Moreover, SPMs reduce the adhesion, transmigration across epithelial cells and generation of reactive oxygen species of neutrophils (Headland and Norling 2015; Romano et al. 2015).

Monocytes and macrophages are also potently regulated by SPMs. The phagocytosis and efferocytosis activities of macrophages are improved by multiple SPM classes representing one of the most prominent proresolving mechanisms of SPMs (Serhan and Levy 2018). Moreover, the chemotaxis and adhesion of monocytes are enhanced by lipoxins (Maddox and Serhan 1996) and the polarisation of macrophages towards an anti-inflammatory phenotype by maresins (Marcon et al. 2013).

In addition to the innate immunity, the cells of the adaptive immunity are regulated by SPMs. RvDs are known to diminish the differentiation of T cells to proinflammatory Th1 and Th17 cells and to reduce the cytokine production of activated Th1, Th17 and CD8+ cytotoxic T cells (Chiurchiu et al. 2016). Moreover, maresins promote the formation of Tregs (Krishnamoorthy et al. 2015), while lipoxins reduce the proliferation and antibody production of B memory cells (Ramon et al. 2014).

SPMs have numerous demonstrated effects, such as counter-regulating the production of proinflammatory cytokines and chemokines (Serhan et al. 2002;

Hong et al. 2003), downregulation of NF-κB gene expression and subsequent proinflammatory cytokine production (Arita et al. 2005), promoting tissue regeneration (Serhan et al. 2012), alleviation of pain (Xu et al. 2010) and enhanced bacterial killing and clearance (Spite et al. 2009; Chiang et al. 2015). Intriguingly, the administration of SPMs has been shown to lower antibiotic requirements for bacterial clearance (Chiang et al. 2012) and to suppress cancer growth (Sulciner et al. 2018), indicating that these molecules have potential indications in the field of medicine.

LMs are autocrine and paracrine molecules that are generally secreted out of the cell. Intriguingly, certain reports describe that EVs can also contain LMs or their pathway markers. EVs derived from human polymorphonuclear neutrophils (PMNs) contained SPM pathway markers (Norling et al. 2011), and PGs have also been detected in the EVs of rat basophilic leukaemia cells (Subra et al. 2010). Remarkably, SPMs have been detected in platelet-derived EVs elucidating that the precursor PUFAs, monohydroxy pathway markers and the SPM end products are all transported via vesicles (Valkonen et al. 2019).

2.3.4 LIPID METABOLISM OF MESENCHYMAL STROMAL CELLS

The ability to biosynthesise lipids, fatty acids and their bioactive derivatives varies from cell type to cell type. For example, hepatocytes are specialised in the lipid metabolism and *de novo* lipid synthesis, and the majority of fatty acid modifications take place in the liver. Due to the pivotal role of lipids and LMs in inflammation, it is crucial to elucidate the lipid metabolism of MSCs to obtain more insight into their immunomodulatory mechanisms.

The phospholipidome of human MSCs has been investigated in three reports demonstrating that the phospholipidome is relatively stable in MSCs derived from different donors (Kilpinen et al. 2013b; Campos et al. 2016; Tigistu-Sahle et al. 2017). As MSCs were passaged during cell culture, their lipid composition changed and the cells with a higher passage number elicited an increased n-6/n-3 PUFA ratio in their phospholipid membranes (Kilpinen et al. 2013b). The GP species containing AA increased during cell culture, whereas the GP species containing DHA decreased. Excitingly, these changes also correlated with a lowered immunosuppressive capacity, which suggests that the changed PUFA content had an impact on the immunomodulation of human MSCs.

Additionally, the phospholipidome of MSCs can be modified with an inflammatory stimulus (Campos et al. 2016) and PUFA supplementation (Tigistu-Sahle et al. 2017). An inflammatory stimulus (MSC licencing cocktail with IFN-γ and TNF-α) resulted in multiple changes in the phospholipid profile (Campos et al. 2016). The relative abundance of PC species containing AA grew with the stimulus. However, the levels of PC and PE species showed a mixed response with DHA-containing species as the relative abundance of PC species with DHA was lowered, while the amount of PE species with DHA

increased. All in all, these results indicate that lipids have a role in the immunomodulatory function of MSCs.

Tigistu-Sahle et al. (2017) have shown that the GP and fatty acid profiles of MSCs can be modified with a 9-day PUFA supplementation with ALA, LA, AA, EPA and DHA. Intriguingly, the 18-carbon PUFA precursors were elongated into 20-carbon PUFAs. However, further processing through desaturation was limited in MSCs. This observation and the gene expression data indicated that the Δ5-desaturation is not efficient in MSCs, which highlights the need for the supplementation of LM-precursor PUFAs, i.e., AA, EPA and DHA, in the cell culture medium. With AA supplementation, the production of PGE_2 was increased, indicating that when the cells have a sufficient supply of the LM precursors, the downstream LMs are produced in higher quantities. More importantly, with PUFA supplementation, it is possible to modify the n-6/n-3 PUFA ratio of the membranes of MSCs, which may be of a great importance in the light of the immunomodulatory capacity of these cells (Kilpinen et al. 2013b; Tigistu-Sahle et al. 2017). Interestingly, it has been reported that EPA and DHA supplementation can also enhance the angiogenic potential of MSCs, thus improving their wound healing properties (Mathew and Bhonde 2018).

MSCs are known to produce PGE_2, which has a central role in their immunomodulatory mechanisms (Aggarwal and Pittenger 2005; Németh et al. 2009; Ylöstalo et al. 2012; Jin et al. 2019). However, otherwise the LM and SPM production of human MSCs has received little attention. The SPM profiles of control-, AA- and DHA-supplemented murine MSCs have been determined, demonstrating that the SPM production is elevated after a carbon monoxide treatment (Tsoyi et al. 2016). Interestingly, the DHA-supplemented MSCs produced more SPMs and promoted mice survival in a sepsis model when compared with the AA-supplemented MSCs. Other studies have demonstrated that EPA-supplemented murine MSCs produce more RvD1 and PGE_2 than control MSCs (Abreu et al. 2018; Silva et al. 2019). Interestingly, these EPA-supplemented MSCs elicited an improved therapeutic response in experimental allergic asthma (Abreu et al. 2018) and sepsis models (Silva et al. 2019). Additionally, it has been demonstrated that LXA_4 is involved in the protective response of murine MSCs in a diabetic nephropathy disease model (Bai et al. 2019). The information on the SPM production of human MSCs remains sparse with only the production of LXA_4 having been reported and shown to partly mediate the resolution of acute lung injury in mice (Fang et al. 2015).

The lipidomics of EVs is challenging due to the small amount of sample material and thus, a large volume of cells is typically required to obtain EVs in sufficient amount for the analysis. Despite these challenges, the lipidome of human MSC-EVs has been fully characterised (Vallabhaneni et al. 2015; Haraszti et al. 2016; Showalter et al. 2019). The profiles of GP classes, sphingolipids, glycolipids, triacylglycerols, diacylglycerols, acylcarnitines and free fatty acids of MSC-EVs have been reported. In general, the lipids of the cell membrane and the secreted EVs typically resemble each other. However,

certain changes arise most likely from the differences in the membrane curvature and signalling pathways between cells and EVs (Skotland et al. 2020). SPMs, their pathway markers and traditional LMs have been identified from EVs derived from other cell sources (Subra et al. 2010; Norling et al. 2011; Valkonen et al. 2019), but the SPM profile of MSC-EVs remains to be investigated.

There is a growing body of knowledge on the lipidome of MSCs with an increasing interest towards the SPM signalling. The reports on the lipid metabolism of MSCs highlight the importance of lipids in the functioning of MSCs. Moreover, because of the ability of MSCs to modulate immune cells towards a phenotype promoting host defence and attenuating inflammation, it has been proposed that MSCs can promote the resolution of inflammation (English 2013). This paradigm opens new avenues in the research on the immunomodulatory mechanisms of MSCs from the perspective of resolution, proresolving lipids and LMs. Taken together, the aforementioned interesting reports suggest that phospholipids and the resulting lipid metabolism have a significant role in the functioning and immunomodulation of MSCs.

3 AIMS OF THE STUDY

The lipid metabolism and EV biology are vital to the immunomodulation of MSCs. The investigation of new immunomodulatory mechanisms of action is important to understand and eventually to enhance MSC therapeutic potential. Moreover, further research on EVs is important in their application as therapeutic agents. Our vision is to elucidate novel ways to improve the immunomodulation, and ultimately the therapeutic potential, of MSCs and MSC-EVs via PUFA supplementations.

The aim of this thesis was to investigate the immunomodulatory mechanisms of human bone marrow-derived MSCs (hBMSCs) and hBMSC-EVs. We focused on the effects of hBMSCs and hBMSC-EVs on immunoregulatory macrophages. Moreover, we examined whether hBMSC and hBMSC-EV membranes can be modified with PUFA supplements to enhance their immunomodulatory response.

The specific aims of this thesis were to:

I. Elucidate how hBMSCs and hBMSC-EVs affect the phenotype of immunoregulatory macrophages and the role of bioactive LMs in the process.

II. Investigate the effects of PUFA supplements on the lipid metabolism of cellular membranes and LM production of hBMSCs and hBMSC-EVs.

III. Investigate the impact of PUFA-modified membranes on the hBMSC immunomodulation mediated via the secretome and cell-cell contact in a coculture assay with immunoregulatory macrophages.

4 MATERIALS AND METHODS

A simplified list of the experimental methods used in this thesis is collated in **Table 1**. The materials and methods are described in more detail in the original publications.

Table 1 Methods employed in the thesis.

Experimental method	Study
Cell culture	I, II and III
PUFA supplementation	II and III
Coculture assay with macrophages	I and III
Phagocytosis assay with *Candida albicans*	III
Isolation of extracellular vesicles	I and II
Nanoparticle tracking analysis	II
Immunoblotting	II
Cytokine determination	I and III
Quantitative polymerase chain reaction	III
Flow cytometry	I and III
Imaging flow cytometry	III
Mass spectrometric analysis of phospholipids	II
Gas chromatographic analysis of fatty acids	II
Mass spectrometric analysis of lipid mediators	I and II
Statistical analysis	I, II and III

PUFA, polyunsaturated fatty acid.

4.1 ETHICAL CONSIDERATIONS

The patient protocols considering the isolation of hBMSCs were approved by the Ethical Committee of Northern Ostrobothnia Hospital District or the Ethical Committee of the Hospital District of Helsinki and Uusimaa. Aspirates of the bone marrow were collected from the upper femur metaphysis or iliac crest of adult patients after receiving a written informed consent. The hBMSCs were isolated and characterized as described previously (Leskelä et al. 2003; Peura et al. 2009). The utilization of anonymised peripheral blood mononuclear cells (PBMCs) from voluntary blood donors in research is in accordance with the rules of the Finnish Supervisory Authority for Welfare and Health (Valvira).

4.2 CELL CULTURE

4.2.1 HUMAN BONE MARROW-DERIVED MSC CULTURE

Primary hBMSCs were thawed and seeded at a density of 1,000-1,200 cells/cm² onto 10 cm or 15 cm plates (Nunclon™ Delta Surface, Thermo Fisher Scientific) using proliferation medium: α-minimum essential medium (MEM) supplemented with 10% foetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin and 20 mM HEPES (all from Thermo Fisher Scientific). The cells were cultured at 37 °C in a 5% CO_2 humidified incubator until 80% confluence, detached with TrypLE™ Express (Thermo Fisher Scientific) and seeded into further experiments. The cell number and viability were calculated using a NucleoCounter® NC-100™ (ChemoMetec). hBMSCs derived from two donors were employed in Study I, four donors in Study II and three donors in Study III.

4.2.2 POLYUNSATURATED FATTY ACID SUPPLEMENTATION

After reaching 80–90% confluence, hBMSCs were supplemented with PUFAs: AA, EPA or DHA (all from Cayman Chemical). First, the 10% FBS-containing proliferation medium was replaced with medium containing only 5% FBS to limit the available fatty acids in the medium. PUFAs were bound to bovine serum albumin (fatty acid-free, Sigma-Aldrich) at a ratio of 2.7:1 PUFA/bovine serum albumin. The final PUFA concentration in cell culture medium was 50 µM. Ethanol (Altia) was used as control as the PUFAs were diluted in ethanol.

4.2.3 EXTRACELLULAR VESICLE ISOLATION AND CHARACTERISATION

To ensure a sufficient yield of EVs, hBMSCs were cultured in two-chamber type of Corning® CellSTACK® cell culture chambers (Sigma-Aldrich) until 80-90% confluence. The cells were then washed three times with phosphate buffered saline (PBS) and once with α-MEM to remove FBS and FBS-derived EVs and then incubated in plain α-MEM for 48 h. The EVs were collected from the conditioned medium via ultracentrifugation. The medium was first centrifuged at 2,000 g for 10 min to remove cell debris. The supernatant was ultracentrifuged at 100,000 g for 2 h at 4 °C using an MLA-50 rotor (k-factor = 92, Beckman Coulter) and Optima™ MAX-XP Ultracentrifuge (Beckman Coulter). In Study I, the pelleted EVs were combined and added into the coculture experiment with macrophages without further washes. The EVs were derived from two hBMSC donors. In Study II, EVs were combined and further ultracentrifuged at 100,000 g for 2 h at 4 °C using an MLS-50 rotor (k-factor = 71, Beckman Coulter) to remove possible lipoproteins (floating in the supernatant rather than pelleting to the bottom) before these samples were

subjected to western blot and mass spectrometric phospholipid analysis. These EVs were derived from three different hBMSC donors.

The size distribution and the quantity of particles in the EV samples were characterised by nanoparticle tracking analysis (NTA) as described in Study II. Moreover, the protein levels of the EV samples were analysed with Western blot as described in Study II. The antibodies against CD9 (clone M-L13, Becton Dickinson), CD41 (clone sz22, Beckman Coulter), CD63 (clone H5C6, Becton Dickinson), CD73 (ab124725, Abcam), cytosolic PLA_2 (ab58375, Abcam) and secretory PLA_2 (ab23705, Abcam) were investigated.

4.2.4 COCULTURE ASSAY WITH MACROPHAGES

Human PBMCs were obtained from buffy coats of voluntary blood donors. The PBMCs were isolated with Ficoll density gradient as described in Studies I & III and 2-4 x 10^6 cells were seeded on either 24- (Study I) or 12-well plates (Study III). The PBMCs were allowed to attach for 1-1.5 h, washed with PBS and the attached monocytes were cultured in 1640 RPMI medium supplemented with 10% FBS (Sigma), GlutaMAX™ and growth factors depending on the macrophage polarisation. In Study I, monocytes were polarised and activated towards M1, M2 and Mreg phenotypes to characterise Mregs. In Studies I and III, the effects of hBMSCs were investigated with macrophages polarised towards the Mreg phenotype. In both studies, each replication was conducted with PBMCs derived from different buffy coat donors.

The layout of the 10-day macrophage polarisation assay is described in **Figure 6**. In Study I, the macrophages were both polarised with 5 ng/mL macrophage colony-stimulating factor (M-CSF) and activated with 25 ng/mL IFN-γ and 10 ng/mL LPS towards Mreg phenotype (termed mature Mregs or Mreg-activated macrophages). In Study III, the macrophages were either polarised with 5 ng/mL M-CSF (termed Mreg-polarised macrophages) or polarised and activated as in Study I (mature Mregs or Mreg-activated macrophages). The cells were activated at day 6 and hBMSCs were added to the coculture at day 7 and incubated for 3 days. In Study I, the hBMSCs were cocultured in cell-cell contact with macrophages and hBMSC-EVs were added in two doses on days 7 and 9. In Study III, control-, DHA- and AA-supplemented hBMSCs were cocultured with both cell-cell contact and on inserts (Corning™ Transwell™, pore size 0.4 μm, polyester membrane, Fisher Scientific) to determine the effect of the hBMSC secretome. At day 10, the cells were detached with 4 °C Macrophage Detachment Solution DFX (PromoCell) for flow cytometry analysis or scraped with RLT lysis buffer (Qiagen) containing 1% 2-mercaptoethanol (Merck) for mRNA analysis.

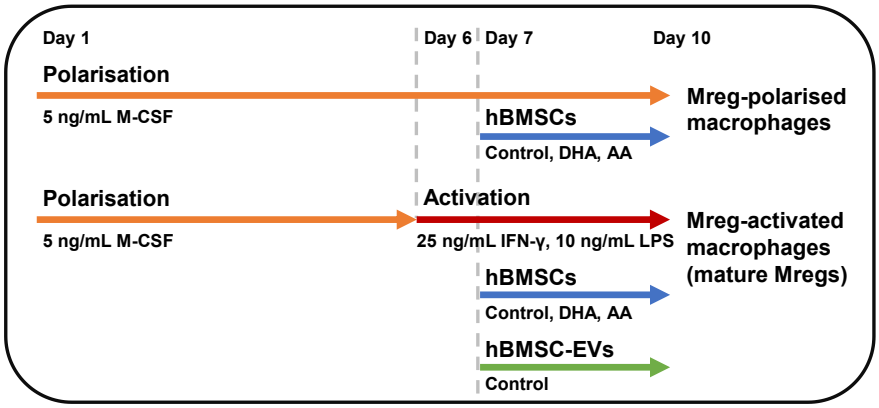

Figure 6 **The layout of macrophage polarisation assay employed in Studies I and III.** Mreg, regulatory macrophage; hBMSC, human bone marrow-derived mesenchymal stromal cell; EV, extracellular vesicle; DHA, docosahexaenoic acid; AA, arachidonic acid; M-CSF, macrophage colony-stimulating factor; IFN; interferon; LPS, lipopolysaccharide. Figure adapted from Figure 1 in Study III.

4.2.5 PHAGOCYTOSIS ASSAY

Candida albicans yeast cells were heat-killed and stained with carboxyfluorescein succinimidyl ester (CFSE) as described in Study III. At day 10 of the macrophage assay, the stained *C. albicans* cells at the concentration of 5×10^5 cells/well (low) or 1.25×10^6 cells/well (high) were added to the Mreg-polarised macrophages with/without hBMSC coculture on inserts. After a 45-min incubation at 37 °C, 5% CO_2, the cells were detached and analysed with imaging flow cytometry. PBMCs derived from five different buffy coat donors and three hBMSC donors were employed in this experiment.

4.3 PHENOTYPIC CHARACTERISATION

4.3.1 CYTOKINE DETERMINATION

The cell culture medium samples from macrophage experiments were analysed with different assays to determine changes in the cytokine production due to hBMSC cell-cell contact, secretome or hBMSC-EVs. In Study I, the coculture medium was analysed for 18 cytokines using human Th1/Th2/Th9/Th17/Th22/Treg 18 Plex ProcartaPlex Immunoassay (Thermo Fisher Scientific) according to the manufacturer's protocol and Luminex instrument platform. In Study III, the coculture medium was analysed with Human TNF-alpha, IL-10 and IL-23 DuoSet enzyme-linked immunosorbent assays (ELISA, all from R&D Systems) according to the manufacturer's protocol.

4.3.2 REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION

In Study III, macrophages with hBMSC secretome samples (hBMSCs cultured on an insert above macrophages) were analysed with real time quantitative polymerase chain reaction (QPCR). RNA was extracted and converted into cDNA as described in Study III. The gene expression was analysed with real-time QPCR (CFX96™ Real-Time Systems &C1000™ Thermal Cycler, Bio-Rad, XX) using TaqMan® Gene Expression assays and TaqMan® Universal Master Mix II with uracil-N-glycosylase (all from Applied Biosystems) as described in Study III. The assays were for genes TGFB1 (ID: Hs00998133_m1), MMP2 (ID: Hs01548727_m1), DHRS9 (ID: Hs00608375_m1), STAT3 (ID: Hs00374280_m1) and STAT1 (ID: Hs01013996_m1), and all assays were equipped with FAM dye and minor groove binder quencher. HPRT1 was used as the reference gene. All samples were analysed as duplicates and the results were analysed with CFX Manager™ 3.0 (Bio-Rad). The relative gene expression levels are expressed as log2 fold change relative to the Mreg-polarised or Mreg-activated macrophages.

4.3.3 FLOW CYTOMETRY

Cells from coculture assays were detached and analysed with flow cytometry to investigate the expression of cell surface proteins. The cells were suspended in 50 µL staining buffer (0.3% bovine serum albumin, 2 mM EDTA in PBS, pH 7.2) and incubated with 2.5 µg of Human BD Fc Block™ (BD Biosciences) for 10 min at RT. In Study I, the cells were stained with anti-human antibodies PE-CD80 (clone 2D10.4, mouse IgG1 κ, eBioscience), PE-Cy7-CD86 [clone 2331 (FUN-1), mouse IgG1 κ, BD Biosciences], BV421-CD163 (clone GHI/61, mouse IgG1 κ, BD Biosciences) and APC-CD206 (clone 19.2, mouse IgG1 κ, BD Biosciences) according to the manufacturers' protocol. In Study III, the cells were stained with using anti-human antibodies PE-CF594-CD86 (clone 2331, FUN-1, mouse IgG1 κ, BD Biosciences), FITC-HLA-DR (clone L243, mouse IgG2a κ, BioLegend), APC-CD206 (clone 19.2, mouse IgG1 κ, BD Biosciences), BV421-CD163 (clone GHI/61, mouse IgG1 κ, BD Biosciences), BV 510™-CD274 (PD-L1, B7-H1) (clone 29E.2A3, mouse IgG2b κ, BioLegend), PE/Cy7-CD120b (TNFR2, clone 3G7A02, rat IgG2a κ, BioLegend), PE-MERTK (clone HMER5DS, mouse IgG1 κ, eBioscience™, Thermo Fisher Scientific) and PerCP-Cy™5.5-CD90 (clone 5E10, mouse IgG1 κ, BD Biosciences). Cell viability was determined with LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit (for 633 or 635 nm excitation, Thermo Fisher Scientific). Conjugated isotype control antibodies were used as negative control for background staining. The stained cells were incubated on ice for 30 min in darkness. After the staining, the cells were washed with 2 mL staining buffer, centrifuged at 350 g for 10 min and suspended in 100 µL staining buffer.

Cells were analysed with BD FACSAria IIU (BD Biosciences) flow cytometer using FACSDiva™ version 8.0.1 software (BD Biosciences) and analysed with FlowJo® version 10.0.7 software (FlowJo, LLC). Macrophages

were gated based on forward and side scatter patterns. Doublets and aggregates were excluded using forward scatter area versus height. The fluorescence positive cells were gated based on isotype controls and populations. In Study III, the CD90 positive cells, i.e., hBMSCs, were excluded from the analysis.

4.3.4 IMAGING FLOW CYTOMETRY

Cells from the phagocytosis assay were stained as described in **Section 4.3.3**. The used fluorochromes were PE-CF594-CD86 and APC-CD206. The stained cells were analysed with Amnis® ImageStream®X Mark II (Luminex Corporation) imaging flow cytometer as described in Study III. The data was collected and analysed with INSPIRE® software (Luminex Corporation).

4.4 LIPIDOMIC AND METABOLIPIDOMIC ANALYSES

4.4.1 MASS SPECTROMETRIC ANALYSIS OF PHOSPHOLIPIDS

The cells were washed twice with ice-cold PBS, harvested, snap frozen and stored at -70 °C prior to the analysis. Lipids were extracted from cell or EV pellets according to the Folch method (Folch et al. 1957). Extracted samples were evaporated into dryness under gentle N_2 stream and dissolved with chloroform:methanol (1:2) (both from Merck). Internal standards and NH_4OH (final concentration 1%, Merck) were added to the samples prior to direct infusion into the ESI source of 6490 Triple Quadrupole mass spectrometer (Agilent Technologies) at a flow rate of 10 µL/min. Phospholipids were detected and quantified by employing lipid class specific MS/MS scans as follows: precursor ion scan *m/z* 184 for PC and SM, neutral loss of 141 amu for PE and neutral loss of 87 amu for PS (Brügger et al. 1997). Spectra were processed with MassHunter Qualitative Analysis (Agilent Technologies) and phospholipids were identified and quantified with Lipid Mass Spectrum Analysis LIMSA (Haimi et al. 2006). The results are described as mol% of each lipid species in its phospholipid class, and the species exceeding 1.0 mol% are included in the figures.

4.4.2 GAS CHROMATOGRAPHIC ANALYSIS OF FATTY ACIDS

The Folch extracted lipid extracts were derivatised into fatty acyl methyl esters according to the recommendations of William Christie (Christie 1993). With this method, all fatty acids (membrane-bound, lipid droplet- and cytosol-derived fatty acids) are derivatised. The samples were analysed with GC employing GC-2010 Plus (Shimadzu) equipped with a flame ionisation detector to obtain the molar composition of fatty acids. The identities of the

fatty acids were confirmed with GC-2010 Plus with GCMS-QP2010 Ultra (Shimadzu). The results are described as mol% of the total fatty acids.

4.4.3 MASS SPECTROMETRIC ANALYSIS OF LIPID MEDIATORS

The cell culture samples were collected, snap frozen and stored at -70 °C prior to the analysis. The samples were thawed and 2 volumes of ice-cold methanol containing internal standards were added. The samples were incubated at least 45 min in -20 °C to allow protein precipitation. Samples were then centrifuged and the supernatant was either eluted with Captiva ND Lipids filter (Study I) or using solid phase extraction as described previously (Colas et al. 2014) (Study II). The purified LM samples were concentrated and injected into an LC-MS/MS system. In Study I, the instrument 1290 Infinity LC coupled to 6490 MS/MS (Agilent Technologies), located in Helsinki, was employed in the analysis. In Study II, the LC-20AD HPLC (Shimadzu) coupled to 6500+ or 5500 MS/MS (SCIEX), located in London, were employed in the analysis as described previously (Colas et al. 2014; Rathod et al. 2017). Results are expressed as ng/mL (Study I) or as pg/incubation (Study II).

4.5 STATISTICAL ANALYSIS

In all Studies, the results are reported as medians with interquartile ranges or ranges. The statistical significances were assessed with nonparametric analyses employing groupwise tests Kruskal–Wallis test (all Studies) and Jonckheere-Terpstra test for testing trends (Study II). Pairwise testing was conducted with Mann-Whitney *U* test (Study I), Wilcoxon matched-pairs signed-rank test (Study I and III) and *post hoc* Dunn's-test for multiple comparisons (Study III). The statistical testing was performed with GraphPad PRISM® version 7.02 (GraphPad Software, Study I), IBM SPSS Statistics (Version 24, Study II) or R version 3.5.1 and the PMCMR package (Pohlert 2014; R Core Team 2018) (Study III). In all studies, p-values < 0.05 were considered statistically significant. In Study II, the phospholipid and LM data was visualised with principal component analysis (PCA) using R version 3.5.1 with the ggbiplot package (Vu 2011; R Core Team 2018).

5 RESULTS

5.1 PUFA SUPPLEMENTATIONS PROFOUNDLY MODIFY THE LIPIDOME OF MSCS

In Study II, we investigated the effect of PUFA supplementation on the lipid metabolism of hBMSCs from three points of view: 1) the phospholipids of the cell membranes, 2) total fatty acids of the cell and 3) LMs derived from PUFAs. The hBMSCs were supplemented for 2, 6 and 24 hours with PUFAs AA, EPA and DHA, which are precursors of multiple proinflammatory and especially proresolving LMs. The aim of this study was to investigate the extent of changes that we can induce in the lipidome by supplementing PUFAs into the cell culture medium employing gas chromatographic and mass spectrometric analysis methods.

Firstly, all of the supplemented PUFAs incorporated into hBMSC membranes gradually during the 24-hour period (**Figure 7 and Study II: Supplementary Figures 1-3**). However, the dynamics of incorporation varied in different GP classes. There were observable changes in the PC species already after a 2-hour supplementation, with an increasing incorporation after 6 hours and a distinct difference after a 24-hour incubation. The changes in the PC species composition were clear and consistent with the relative increase in 38- to up to 44-carbon PC species. The increase in di-PUFA (two polyunsaturated acyl chains) containing PC species was notably visible after AA and EPA supplementation.

The supplemented PUFAs affected PE and PS remodelling more slowly than the PC species. With PE, we observed some degree of incorporation after 6 hours, however, the most prominent incorporation was visible only after 24 hours. The relative abundance of PE species with 40-carbons or more was most prominently increased at the expense of lipid species with shorter acyl chains and fewer double bonds, notably PE 36:1 and 36:2. In the PS profile, the incorporation was not clearly transferred into the full lipid species profile even after 24 hours. Rather, the incorporation was visible only in the 40-carbon PS species. The sphingolipid SM class remained unaffected by the supplementation, undergoing only minor modifications.

Secondly, the fatty acid profile of hBMSCs evidently confirmed the finding from the GP data, indicating that the PUFA supplementation had been successful (**Study II: Figure 1**). The relative abundance of supplemented PUFAs was notably elevated. Moreover, the 20-carbon PUFAs were readily elongated into their 22-carbon forms: AA (20:4n-6) to adrenic acid (ADA, 22:4n-6) and EPA (20:5n-3) to n-3 DPA (22:5n-3). DHA, being the end product of the n-3 PUFA family, was not further modified. The incorporation of the elongated PUFAs into membrane GPs was evident in the increased relative abundance of 40-carbon lipid species, which consist of the acyl chains

of the 22-carbon elongated forms (i.e., ADA 22:4n-6 and n-3 DPA 22:5n-3) and 18:0 fatty acid. The relative abundance of shorter and less unsaturated fatty acids such as 16:0, 18:1n-9 and 18:1n-7 were decreased by the PUFA supplementations. Interestingly, the levels of n-3 PUFAs DPA and DHA were diminished by AA supplementation whereas the levels of n-6 PUFAs AA and ADA were lowered by the n-3 PUFA supplementations.

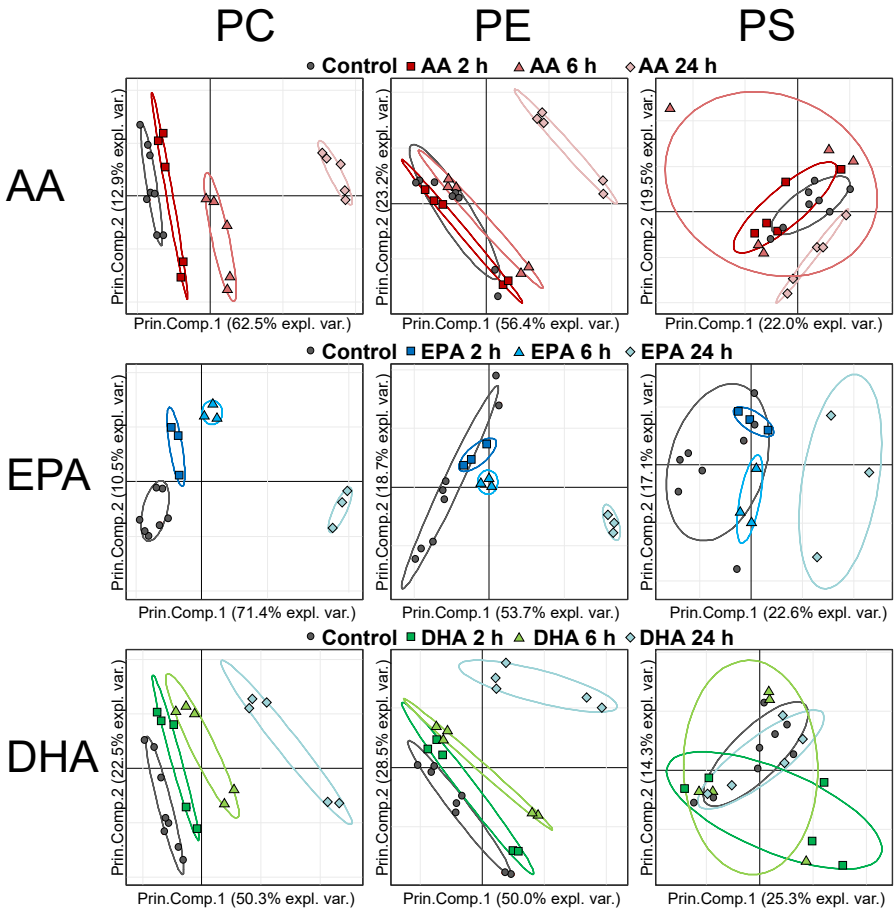

Figure 7 **The incorporation dynamics of supplemented PUFAs differ in hBMSC membrane phospholipid classes.** hBMSCs were supplemented with AA, EPA or DHA for 2, 6 or 24 h and the consequent phospholipidome changes were analysed by ESI-MS/MS. Principal component analysis was conducted for the species profiles of each phospholipid class, n = 8 (control), n = 5 (AA and DHA) or n = 3 (EPA) experimental replicates per group. AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; Prin.Comp, principal component; expl. var, explained variance. Figure adapted from Figure 2 in Study II with the permission of Elsevier.

Finally, we successfully determined the LM profiles of hBMSCs, which had not been reported in full before our study. Highlighting the effects of PUFA incorporation into membrane GPs, the LM profiles were altered after 24-hour PUFA supplementation followed by 48-hour incubation in serum-free medium (**Figure 8 and Study II: Supplementary Table 2**). The PUFAs amplified the production of their respective downstream LMs and mono-hydroxy pathway markers. For example, AA supplementation increased the production of PGE_2, PGD_2, 15-epi-LXA_4 and 15-HETE, EPA improved the production of RvE2, 18-HEPE and 15-HEPE, while DHA increased the production of RvD4, RvD6, 17-HDHA and 14-HDHA. Taken together, these findings show that hBMSCs are able to produce biologically relevant amounts of SPMs, suggesting that these LMs and SPMs have an essential role in hBMSC immunomodulation.

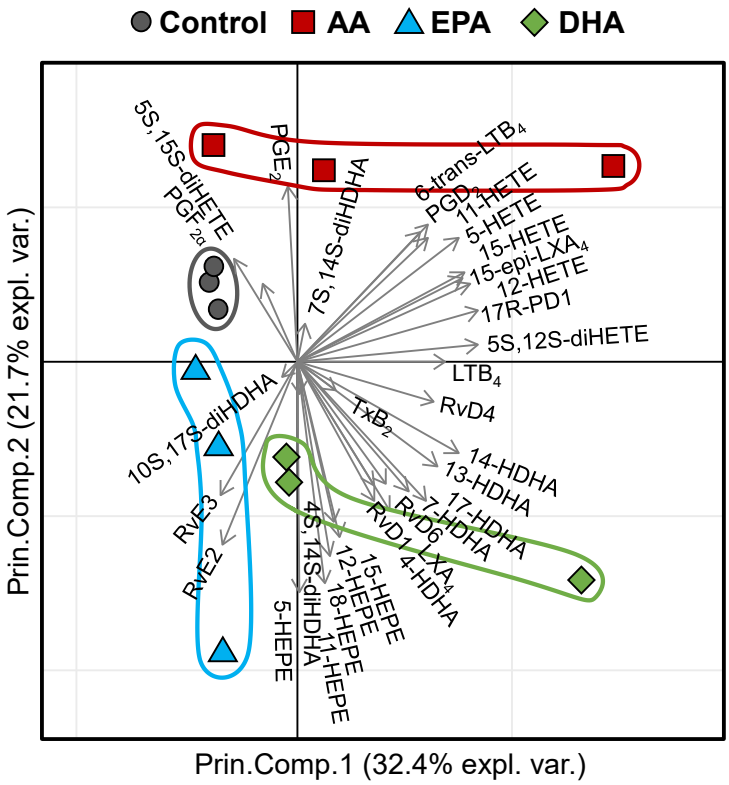

Figure 8 **Incorporated PUFAs alter the downstream lipid mediator profile of hBMSCs.** hBMSCs were supplemented with AA, EPA or DHA for 24 h and then incubated for 48 h in serum-free medium. The effect of the PUFAs on the AA, EPA and DHA metabolomes were investigated with LC-MS/MS and analysed with principal component analysis, n = 3 biological replicates per group. AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; Prin.Comp, principal component; expl. var, explained variance. Figure adapted from Figure 6 in Study II with the permission of Elsevier.

After establishing that hBMSCs produce multiple LMs, we further investigated the effect of anti- and proinflammatory stimuli on the LM production of hBMSCs (**Study II: Figure 7 and Supplementary Table 3**). The stimuli were incubated for 48 hours, which may have dampened their initial effects on LM production at the end of the long incubation period. However, LM production was still altered with the stimuli, especially with a highly proinflammatory stimulus containing TNF-α 10 ng/mL, IL-1β 10 ng/mL and LPS 100 ng/mL. This stimulatory condition resulted in an extremely high increase in PG production and promoted SPM production.

5.1.1 THE PUFA MODIFICATIONS ARE TRANSFERRED TO THE PHOSPHOLIPIDOME OF MSC-EVS

We determined the phospholipid profiles of hBMSCs-EVs with and without PUFA supplementation. The hBMSCs were first supplemented with PUFAs for 24 hours and then incubated in serum-free medium for 48 hours. The long incubation in serum-free medium was conducted to obtain a sufficient EV yield for the mass spectrometric analysis and to remove EVs derived from FBS to allow the investigation of a pure hBMSC-EV fraction. To our delight, we observed that the PUFA incorporation into hBMSC plasma membranes was also transferred into hBMSC-EV membranes (**Figures 9 and 10**). To our knowledge, we showed for the first time that EV membranes can be modified by supplementing cells with PUFAs. Additionally, we demonstrated that the initial 24-hour supplementation and resulting GP modifications are still present in the cell membranes even after 48-hour incubation in serum-free medium.

Even though the EV phospholipid profile resembled that of the cells, we observed certain differences between cells and EVs with all PUFA supplements. The most notable difference was the larger relative abundance of PE and PS species 36:1, containing 18:0 and 18:1 acyl chains, in EVs. The accumulation of MUFA-containing acyl chains in EVs has been demonstrated in previous reports (reviewed by Skotland et al., 2017). Particularly, 18:1 acyl chains accumulate in EVs, which is supported by our results. We also detected an increase in the relative abundance of di-PUFA species (containing two polyunsaturated acyl chains) in the EV fraction. Additionally, we investigated the effect of PUFA supplementation on the number of particles or their size distribution with NTA. These parameters were not altered by PUFA supplementation (**Study II: Figure 3**).

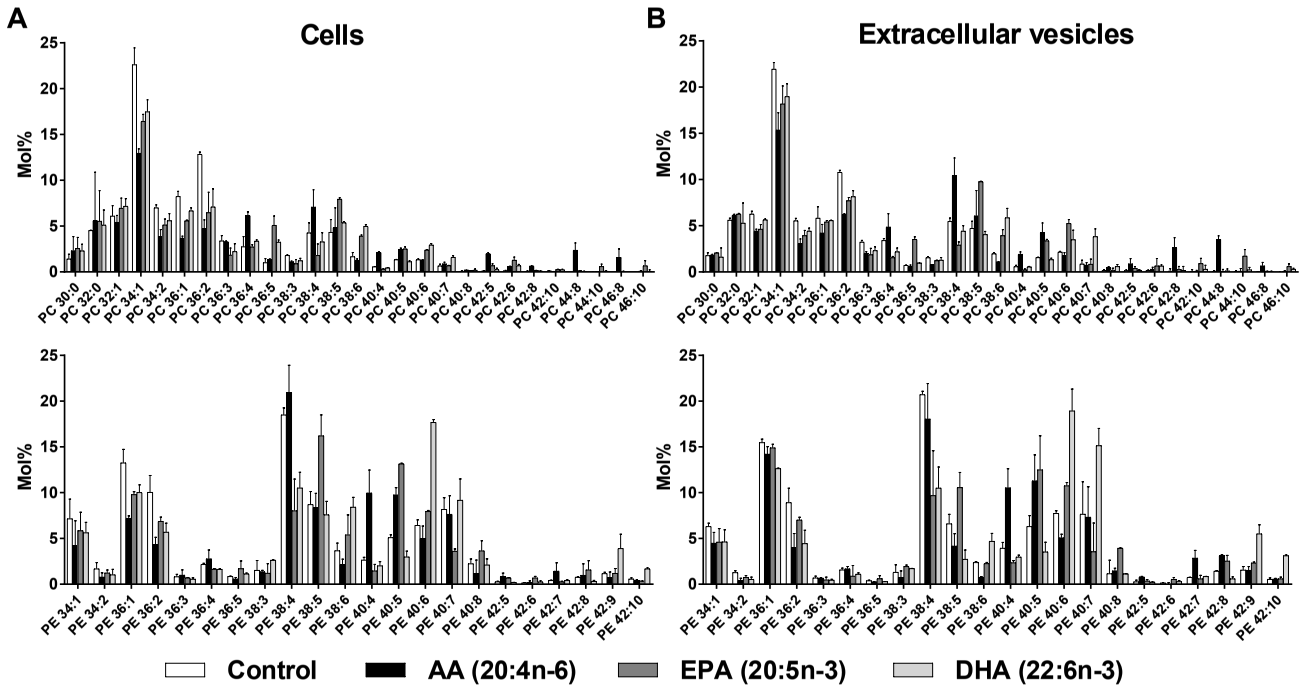

Figure 9 **The PUFA modifications of hBMSC phosphatidylcholine and phosphatidylethanolamine species are transferred to hBMSC-EVs**. Cells were supplemented with AA, EPA or DHA for 24 h and then incubated for 48 h in serum-free medium. The hBMSC-EVs were collected from the cell culture medium via ultracentrifugation. The phospholipid profiles of hBMSCs **(A)** and hBMSC-EVs **(B)** were analysed with ESI-MS/MS. The results are expressed as medians with ranges and as molar percentages (mol%), showing phospholipid species > 1 mol%; n = 3 biological replicates per group. AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine. Figure adapted from Figure 4 in Study II with the permission of Elsevier.

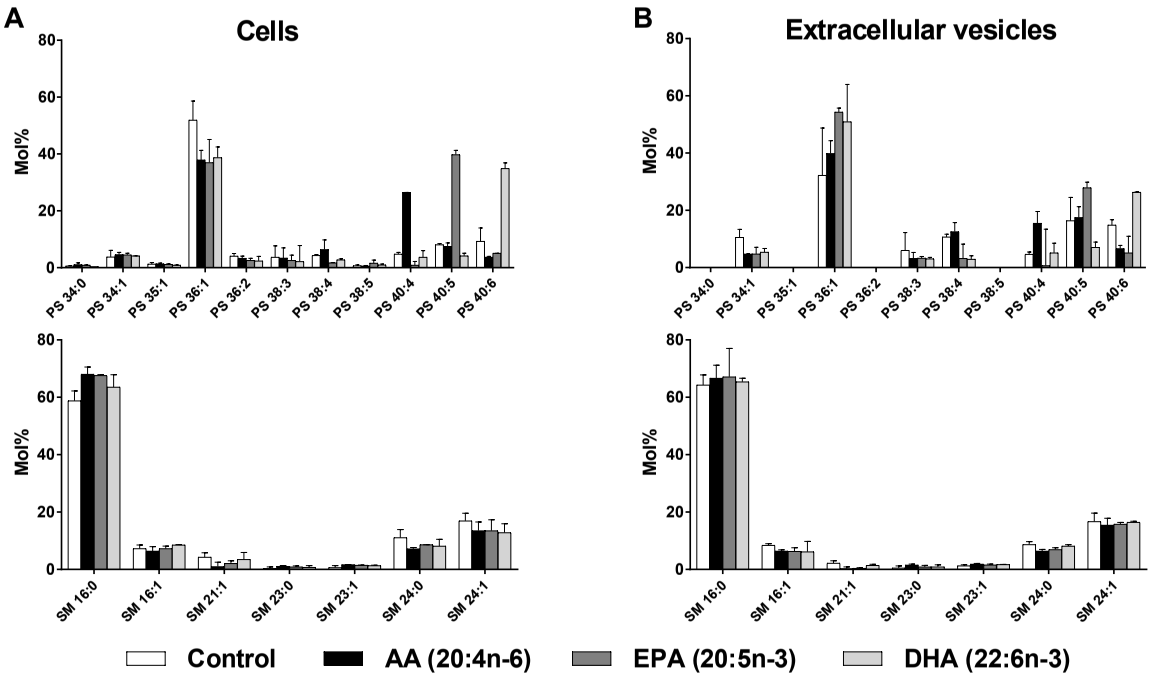

Figure 10 **The PUFA modifications of hBMSC phosphatidylserine species are transferred to hBMSC-EVs**. Cells were supplemented with AA, EPA or DHA for 24 h and then incubated for 48 h in serum-free medium. The hBMSC-EVs were collected from the cell culture medium via ultracentrifugation. The phospholipid profiles of hBMSCs **(A)** and hBMSC-EVs **(B)** were analysed with ESI-MS/MS. The results are expressed as medians with ranges and as molar percentages (mol%), showing phospholipid species > 1 mol%; n = 3 biological replicates per group, except for PS AA n = 2. AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PS, phosphatidylserine; SM, sphingomyelin. Figure adapted from Figure 4 in Study II with the permission of Elsevier.

5.2 MSCS POLARISE REGULATORY MACROPHAGES TOWARDS AN ANTI-INFLAMMATORY PHENOTYPE

To gain more insight into the effects of hBMSC and hBMSC-EV immunomodulation, we examined their effects on macrophages. In Studies I and III, we employed a 10-day macrophage assay to investigate the effects of hBMSCs, hBMSC-EVs and hBMSC secretome on macrophages polarised towards Mregs. First, we developed a macrophage polarisation assay to polarise PBMC-derived monocytes towards M1, M2 and Mreg phenotypes in Study I. The phenotypes of these three differentially polarised macrophage subtypes differed significantly in their cell surface protein expression and cytokine production, although there was variation in the phenotypes of different buffy coat donors (**Figure 11 and Study I: Figures 1-2**). As is typical of the host defence M1-type macrophages *in vitro*, we observed a high expression of cofactor CD86 required for T-cell activation and a high production of proinflammatory cytokines, such as TNF-α, IL-1β, IL-2, IL-5, IL-22 and IL-23 (Broichhausen et al. 2012; Vogel et al. 2014). Moreover, macrophages polarised towards alternatively activated, wound-healing M2-phenotype exhibited typical *in vitro* characteristics with a high expression of mannose receptor CD206, low production of proinflammatory cytokines and high production of anti-inflammatory IL-10 (Vogel et al. 2014).

The phenotype of macrophages polarised towards immunoregulatory Mregs was distinct from the phenotype of the two other macrophage types. These Mregs expressed low levels of CD80 and CD163 and high levels of CD86. Moreover, Mregs produced intermediate levels of multiple proinflammatory cytokines, such as TNF-α, IL-1β, IL-2, IL-22 and IL-23, and very high levels of IL-10. All of the aforementioned observations are in line with typical characteristics of Mregs (Hutchinson et al. 2011). In Study III, our observation that these cells were also positive for HLA-DR and stably expressed the gene for a human Mreg marker dehydrogenase/reductase 9 (DHRS9) is consistent with previous reports (Hutchinson et al. 2011; Riquelme et al. 2017).

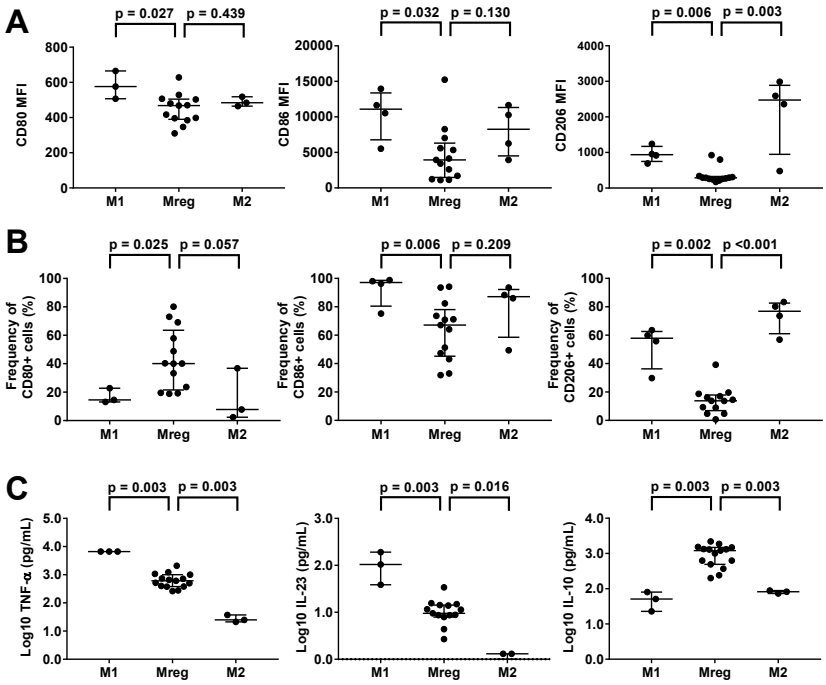

Figure 11 **Phenotypes of M1, M2 and Mreg macrophages.** The cell-surface protein expression of macrophages was examined with flow cytometry and the cytokine production with a Luminex-based 18-panel cytokine assay. The median fluorescence intensities are described in panel **(A)**, the frequencies of positive cells in panel **(B)** and cytokine production in panel **(C)**. The values of cell culture medium cytokines were log10-transformed. The significance in pairwise variation between M1 and Mreg, and M2 and Mreg was analysed using the Mann-Whitney *U* test, and the results are expressed as medians with interquartile ranges. The number of biological replicates varied from 3 to 13 (cell surface proteins) and 15 (cytokines). M1, classically activated "host defence" macrophage; Mreg, regulatory macrophage; M2, alternatively activated "wound healing" macrophage; MFI, median fluorescence intensity; TNF, tumour necrosis factor; IL, interleukin. Figure adapted from Figures 1-2 in Study I.

In Study I, we investigated the effect of hBMSC cell-cell contact and hBMSC-EVs on mature Mregs. Both hBMSCs and hBMSC-EVs had an anti-inflammatory impact on the LM and cytokine production in the coculture (**Figure 12**). In more detail, both the cells and EVs significantly increased PGE_2 levels and reduced the levels of IL-22 and IL-23 in the cell culture medium. Other cytokines were not significantly altered, although there was a decreasing trend in the TNF-α levels. The concentrations of 15-HETE, the pathway marker of lipoxins, and 17-HDHA, the pathway marker of RvDs and protectins, were also greater in the cell culture medium in the Mreg and hBMSC coculture than in the Mreg medium alone, but the EVs had no effect. The expression of cell surface markers was also altered by hBMSCs and

hBMSC-EVs (**Study I: Table 2**). The cells decreased the expression of CD163 and CD206 of Mregs. The EVs also reduced CD163 expression, had a slight effect on CD206 expression and increased CD86 expression. Moreover, we assessed the effect of hBMSC coculture on the phagocytosis activity of Mregs using latex beads coated with FITC-labelled rabbit IgG. However, the hBMSC cell-cell contact had no impact on the phagocytosis activity of Mregs (**Study I: Figure 5**).

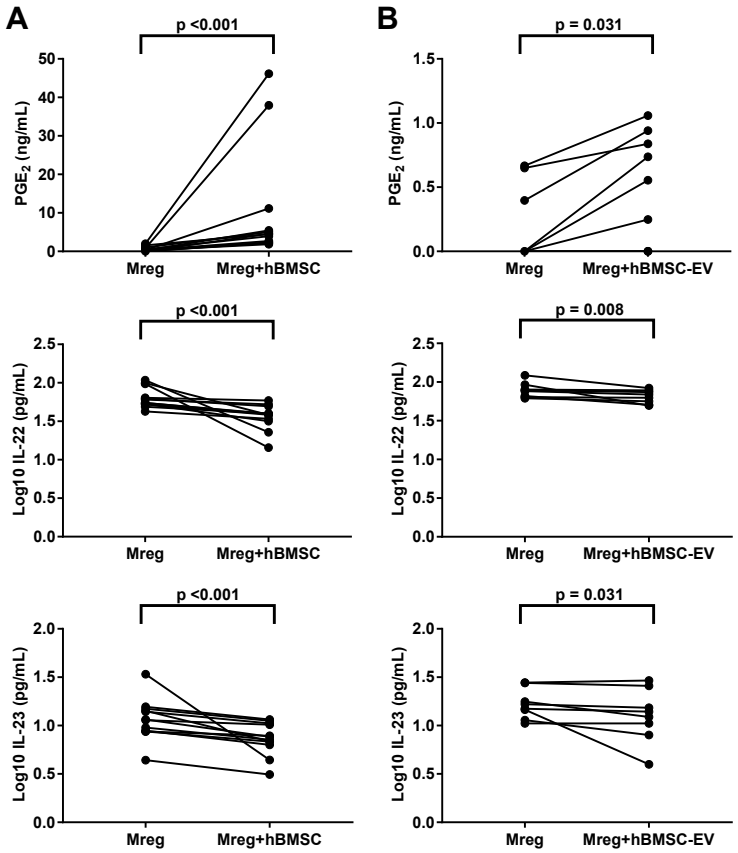

Figure 12 hBMSC cell-cell contact and EVs enhance the anti-inflammatory properties of Mregs. Effect of hBMSC cell-cell contact **(A)** and hBMSC-EVs **(B)** on the level of prostaglandin E_2 and cytokines in Mreg-conditioned medium. The media were analysed with LC-MS/MS to identify lipid mediators and with a Luminex-based 18-panel cytokine assay. The values of cell culture medium cytokines were log10-transformed. The variation between Mreg-conditioned medium with and without hBMSC cell-cell contact or hBMSC-EVs was analysed by the Wilcoxon matched-pairs signed-rank test. The number of biological replicates is 12 for the MSC cell-cell contact experiments and 8 for the EV experiments. Mreg, regulatory macrophage; hBMSC, human bone marrow-derived mesenchymal stromal cell; EV, extracellular vesicle; PGE_2, prostaglandin E_2; IL, interleukin. Figure adapted from Figures 3-4 and Table 3 in Study I.

In Study III, we investigated in more detail how hBMSC cell-cell contact and secretome affected the Mreg phenotype. In contrast to Study I, we examined additional phenotype markers that would pinpoint changes related to the proresolving and wound-healing properties of the macrophages. We polarised macrophages towards the Mreg phenotype and studied both Mreg-polarised macrophages (macrophages only polarised with 5 ng/mL M-CSF) and Mreg-activated macrophages (macrophages polarised with 5 ng/mL M-CSF and activated with 25 ng/mL IFN-γ and 10 ng/mL LPS), the latter of which corresponded to the mature Mregs investigated in Study I.

Particularly, the hBMSC secretome altered the expression of several macrophage cell surface phenotype markers (**Study III: Figures 2-3**). The hBMSC secretome drastically increased the CD206 expression of both Mreg-polarised and Mreg-activated macrophages (**Figure 13**). Moreover, the secretome induced additional anti-inflammatory changes by increasing the PD-L1 expression in Mreg-polarised macrophages and Mer receptor tyrosine kinase (MerTK) expression in Mreg-activated macrophages.

The hBMSC cell-cell contact only slightly affected the phenotype of macrophages by decreasing HLA-DR expression in both macrophage types studied. The different coculture settings had no effect on the cytokine production detected in the cell culture medium (**Study III: Table 1**). Neither was the gene expression of macrophage phenotype markers altered by control-hBMSC secretome (**Study III: Figure 5**). The effect of cell-cell contact on gene expression was not investigated due to the presence of hBMSC-derived mRNA in the samples. Overall, the notable donor-specific variation in the phenotype markers of the different buffy coat donors may have concealed certain effects of hBMSCs on macrophages.

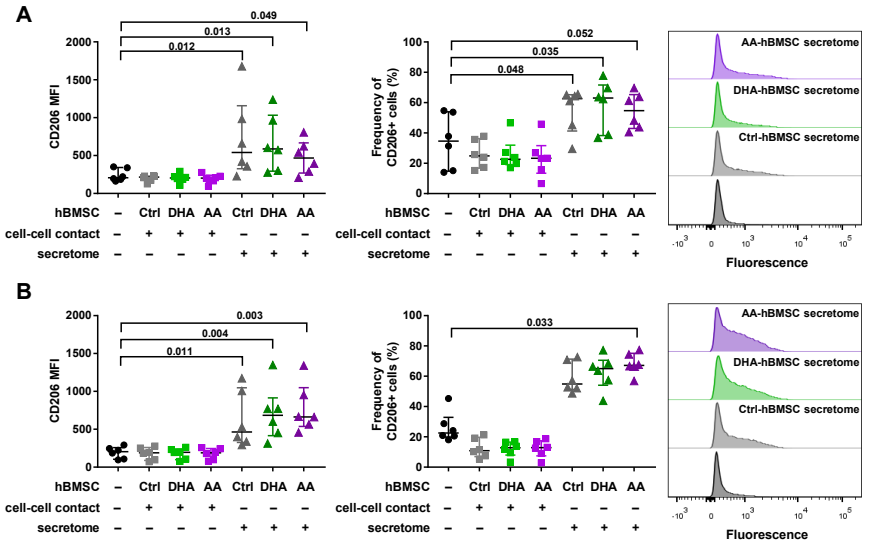

Figure 13 **The secretome of hBMSCs increases the expression of CD206.** The upper panel describes the results of Mreg-polarised macrophages **(A)** and the lower panel the results of Mreg-activated macrophages, i.e., mature Mregs **(B)**. The median fluorescence intensities (left panel) and frequencies of positive cells (middle panel) were determined with flow cytometry analysis. The representative histograms are presented on the right panel. The differences among groups were determined with Kruskal-Wallis rank sum test and *post hoc* using Dunn's-test for multiple comparisons (presented in the figures). The results are expressed as medians with interquartile ranges; n = 6 biological replicates per group. Mreg-polarised, macrophages polarised with 5 ng/mL M-CSF; Mreg-activated, macrophages polarised and activated with 5 ng/mL M-CSF, 25 ng/mL IFN-γ and 10 ng/mL LPS; Ctrl, control; DHA, docosahexaenoic acid; AA, arachidonic acid; MFI, median fluorescence intensity. Figure replicated from Figure 4 in Study III.

We observed that hBMSCs improved the *C. albicans* phagocytosis activity of Mreg-polarised macrophages in a CD206-dependent manner (**Figure 14**). In contrast to our earlier experiments, the induction of CD206 expression was donor-dependent. Thus, we categorised the responders (n = 3 biological replicates) and non-responders (n = 2 biological replicates) based on the fold change of the CD206 expression with Mreg-polarised macrophages with and without hBMSC secretome (**Study III: Supplementary Figure 3**). In the responder group, the more yeast had been added to the cell culture, the more *C. albicans* had been ingested. Moreover, the phagocytosis activity was increased by the hBMSC secretome by 1.6 to 5.8-fold with a low concentration (5 x 10^5 yeast cells/well) of *C. albicans* and by 1.2 to 3.1-fold with a high concentration (1.25 x 10^6 yeast cells/well) of *C. albicans*.

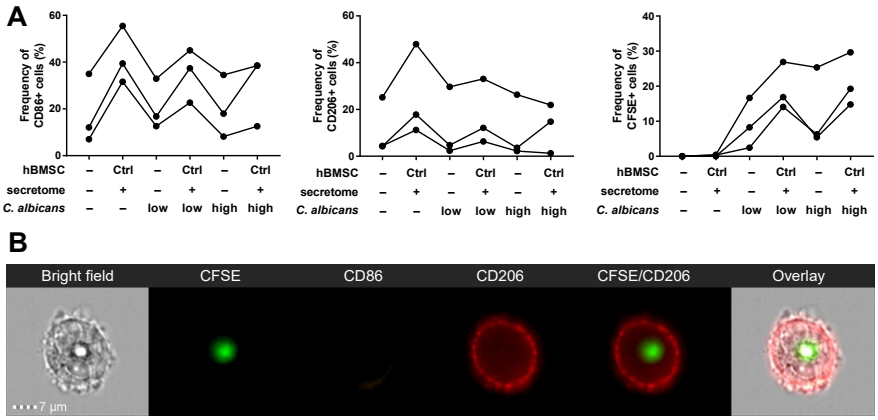

Figure 14 **hBMSC secretome improves the phagocytosis activity of *C. albicans* in Mreg-polarised macrophages in a CD206-mediated manner.** The expression of CD86 and CD206 positive cells and the phagocytosis of CFSE-stained *C. albicans* was determined with imaging flow cytometry **(A)**. A representative imaging flow cytometry image of a macrophage cell that has phagocytosed *C. albicans* **(B)**. hBMSC, human bone marrow-derived mesenchymal stromal cell; low *C. albicans* concentration, 5 x 10^5 yeast cells/well; high *C. albicans* concentration, 1.25 x 10^6 yeast cells/well; CFSE, CFSE-stained *C. albicans*. Figure replicated from Figure 6 in Study III.

5.2.1 PUFA SUPPLEMENTATIONS HAVE A LIMITED IMPACT ON MSC IMMUNOMODULATION

To determine the effects of PUFA supplementation, and the consequent enhanced SPM production, on hBMSC immunomodulation, we examined the effect of hBMSCs supplemented with DHA or AA (DHA-hBMSCs or AA-hBMSCs, respectively) on macrophages. The main differences in the macrophage phenotype were induced by the hBMSC secretome regardless of the PUFA supplementation. The control- and PUFA-supplemented hBMSCs caused similar phenotype changes with certain differences. Particularly, the secretome of DHA-hBMSCs enhanced the CD163 expression and decreased the gene expression of gelatinase matrix metalloproteinase 2 (MMP-2) in Mreg-polarised macrophages, while control- or AA-hBMSCs had no effect (**Figure 15**). The CD163 expression was variable between individual buffy coat donors and was greatly elevated in two donors by both control- and DHA-hBMSC secretome. However, the DHA-hBMSCs increased the CD163 expression in other donors as well in contrast to the control-hBMSCs. Like DHA-hBMSCs, AA-hBMSCs reduced the MMP-2 gene expression in multiple donors, although the effect was not observed in all donors. The secretome of AA-hBMSCs had no effect on the PD-L1 expression in Mreg-polarised macrophages, while control- and DHA-hBMSCs amplified this expression. Conversely, the increase in CD206 expression in Mreg-activated macrophages was the most prominent with the AA-hBMSC secretome.

The PUFA supplementations had only a limited impact on the hBMSC immunomodulation of Mreg-polarised and activated macrophages. All in all, the DHA-hBMSCs elicited a more pronounced anti-inflammatory effect on macrophages than the other hBMSCs investigated. Yet, clear conclusions on the effects of PUFA supplementations on hBMSC immunomodulation require further studies with Mregs and also in other experimental settings.

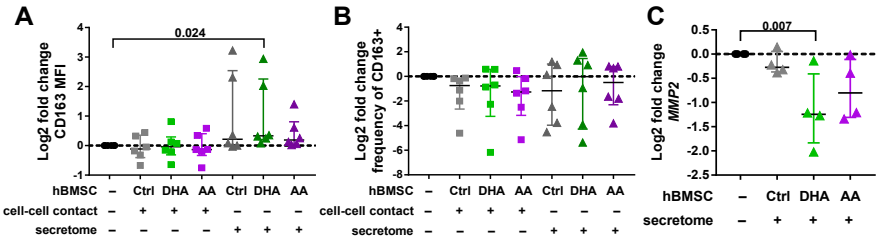

Figure 15 **DHA-supplementation enhances the anti-inflammatory effects of hBMSC secretome on Mreg-polarised macrophages.** The median fluorescence intensity **(A)** and frequency of positive cells **(B)** for CD163 were determined with flow cytometry analysis. The expression of *MMP2* was investigated with QPCR **(C)**. The effect of hBMSCs on Mreg-polarised macrophages is visualised with log2 fold change calculated against macrophages cultured without hBMSCs (represented by the zero line) for each individual donor. The differences among groups were determined with Kruskal-Wallis rank sum test and *post hoc* using Dunn's-test for multiple comparisons (presented in the figures). The results are expressed as medians with interquartile ranges; n = 6 biological replicates for the flow cytometry analysis and n = 4 biological replicates per group for the QPCR analysis. Mreg-polarised, macrophages polarised with 5 ng/mL M-CSF; Ctrl, control; DHA, docosahexaenoic acid; AA, arachidonic acid; MFI, median fluorescence intensity; *MMP2*, gene for matrix metalloproteinase 2. Figure adapted from Figures 2 and 5 in Study III.

6 DISCUSSION

6.1 MSC LIPID METABOLISM IS MODIFIED VIA PUFA SUPPLEMENTATION

The lipid metabolism of MSC has an important role in the immunomodulation of MSCs. This concept is reflected in LM production, particularly with regards to PGE_2 and the newly identified SPMs, and the correlation of the membrane fatty acid composition to the immunosuppressive capacity of MSCs (Aggarwal and Pittenger 2005; Kilpinen et al. 2013b; Fang et al. 2015; Bai et al. 2019). Via PUFA supplementation, we aimed to modify the phospholipid, fatty acid and LM profiles of hBMSCs. Moreover, we aimed to investigate whether these changes were transferred to the immunomodulatory capacity of hBMSCs to find ways to promote the therapeutic potential of MSCs (the immunomodulatory changes are discussed in **Section 6.2.4**). Indeed, the PUFA supplements profoundly modified the lipidome and metabolipidome of hBMSCs.

First, we investigated the hBMSC phospholipid profile with ESI-MS/MS, and observed modifications after 2-, 6- and 24-hour PUFA supplementation. Interestingly, the dynamics of the PUFA incorporation varied in different lipid classes, being the fastest with PCs, then PEs and finally with PSs. During the 24-hour supplementation, only limited changes in the PS profile were visible. However, when the phospholipid profile of hBMSCs was inspected after 24-hour PUFA supplementation followed by 48-hour incubation in serum-free medium, the incorporation was more prominent in the PS profile than with the 24-hour supplementation alone. Our previous report on hBMSC PUFA supplementation showed that the PC, PE and PS classes are clearly remodelled after a 9-day PUFA supplementation (Tigistu-Sahle et al. 2017). Moreover, our results are in agreement with previous pulse-labelling studies with mammalian cells, indicating that the phospholipid modification is the fastest in PC, intermediate in PE and slow in PS and SM (Gallaher et al. 1973; Spector et al. 1980). We did not observe the remodelling of SM species, which was expected since SMs rarely contain long-chain PUFAs as acyl chains.

The differences in the PUFA incorporation rates of GP species may stem from different remodelling pathways. Our results demonstrating that PC is remodelled first are in agreement with findings from metabolic studies showing that in the remodelling Lands cycle, lysoPC acyltransferases transfer the supplemented PUFAs to lysoPC, which serves as the first acceptor (Chilton et al. 1996; Pérez-Chacón et al. 2009). The intermediate rate of PE remodelling may be derived from the function of coenzyme A-independent remodelling enzymes, transacylases, which transfer PUFAs from PC to PE in a process that takes several hours in primary cell lines but occurs in minutes in cancer cell lines (Balsinde et al. 1994; Pérez et al. 2006; Zhang et al. 2012). The slow

remodeling rate of PS was most likely due to transacylases that transferred PUFAs mainly from PC to PS between the 6- and 24-h time points. By this time, the supplemented 20-carbon PUFAs had been elongated, resulting in the incorporation of the 22-carbon PUFAs into PS, yielding the 40-carbon species.

Secondly, the fatty acid composition of hBMSCs was investigated with GC analysis. The incorporation of the supplemented PUFAs and their elongated forms was evident during the 2-, 6- and 24-hour supplementation period. The relative abundance of the 22-carbon elongated forms ADA and n-3 DPA derived from AA and EPA, respectively, were greatly increased after 24 hours. The elongation limits the formation of PGs, LTs and lipoxins derived from the 20-carbon PUFAs due to diminished precursor availability. Moreover, both ADA and n-3 DPA inhibit the activities of COX-1 and COX-2, which are key enzymes in the eicosanoid biosynthesis (Akiba et al. 2000; Zou et al. 2012; Dong et al. 2016). This way, the highly reactive AA is converted into a less reactive ADA to limit AA-mediated signalling. This effect was also visible in the PE species composition because the relative abundance of PE 38:4, containing mostly 18:0_20:4n-6, was not elevated above 20 mol% with AA supplementation, restricting the available AA pool.

In addition to limiting eicosanoid biosynthesis, the elongation of EPA to n-3 DPA amplifies the precursor availability for SPM biosynthesis. n-3 DPA is a precursor to multiple potent SPMs including RvT, $RvD_{n\text{-}3\ DPA}$, $PD_{n\text{-}3\ DPA}$ and $MaR_{n\text{-}3\ DPA}$ families (Dalli et al. 2013, 2015a). Thus, EPA supplementation did not only increase the levels of one, but two SPM precursors, skewing the PUFA profile towards a highly proresolving phenotype. Moreover, the supplementation of both n-3 PUFAs decreased the n-6 PUFA levels whereas AA-supplementation diminished the n-3 PUFA levels. These findings demonstrate well the competitive inhibition between n-3 and n-6 PUFA families that is based on the competing PUFA substrates for the common metabolic pathways (Holman and Mohrhauer 1963).

Finally, we determined with LC-MS/MS how PUFA supplementation affected the LM production of hBMSCs. Remarkably, we established for the first time the SPM profile of human MSCs. As expected, the main LM produced by hBMSCs was PGE_2 due to its central role in MSC immunomodulation (Aggarwal and Pittenger 2005; Németh et al. 2009). Moreover, PUFA supplementation elevated the production of the downstream LMs: AA induced an increase in PGE_2, PGD_2 and 15-epi-LXA_4 production, while EPA increased RvE2 and DHA increased RvD4 and RvD6. Furthermore, the levels of downstream monohydroxy pathway markers were elevated. The rise in SPM production as a result of expanding the precursor pool indicates that hBMSCs readily produce these proresolving mediators when precursors are available. Moreover, it emphasises the significance of membrane PUFAs and their derivatives as a potential novel mechanism of action in hBMSC immunomodulation.

The overall level of SPM production by hBMSCs was similar to that of unstimulated murine MSCs (Tsoyi et al. 2016). These levels were lower than

the typical SPM levels produced by human phagocytes (Dalli and Serhan 2012). However, SPMs are highly bioactive molecules and can exert their effects at pico- to nanomolar concentrations, indicating that the levels detected from MSCs are physiologically relevant. Moreover, the SPM production may not reflect the main immunomodulatory mechanism of MSCs, when compared with IDO- and PGE_2-mediated effects, for example. Rather, SPM production could represent an augmenting mechanism of action in the MSC function that could become more prominent with increased PUFA precursor availability.

We observed that stimulatory conditions had an impact on the hBMSC LM production by increasing SPM production. Our finding is in agreement with previous reports demonstrating that licencing with proinflammatory stimuli enhances the immunomodulatory functions of MSCs (Krampera 2011; Noronha et al. 2019). There may be an association between the enhanced immunomodulation observed in other reports and the elevated SPM production of MSCs primed with proinflammatory stimuli, suggesting once more the significant role of these proresolving mediators in the functionality of hBMSCs.

Augmented SPM production after n-3 PUFA supplementation has been associated with the improved therapeutic response of murine MSCs in an allergic asthma and sepsis models (Tsoyi et al. 2016; Abreu et al. 2018; Silva et al. 2019). Moreover, murine MSCs can reverse diabetic nephropathy disease (Bai et al. 2019) and human MSCs attenuate acute lung injury in *in vivo* models via LXA_4 (Fang et al. 2015). SPMs are highly potent molecules in dampening inflammation and modulating the function of immune cells (Serhan and Levy 2018). SPM administration is beneficial in various *in vivo* models, such as in ischemic stroke (Marcheselli et al. 2003), allergic airway inflammation (Haworth et al. 2008) and sepsis (Spite et al. 2009). Indicating a pathophysiological role in their dysregulated biosynthesis, diminished levels of SPMs have been detected in patients with inflammatory diseases, such as severe asthma (Levy et al. 2005) and coronary artery disease (Elajami et al. 2016). Since SPMs have an established role in the dampening of inflammatory responses and human MSCs are now known to produce several SPMs, the SPMs have most likely an important, but not yet fully elucidated, impact on the murine and human MSC therapeutic response.

The cell culture medium is typically devoid of long-chain n-3 PUFAs EPA and DHA (Tigistu-Sahle et al. 2017; Kiamehr et al. 2018). Supplementing MSCs with PUFAs is particularly important due to MSCs' inability to biosynthesise them from 18-carbon ALA (Tigistu-Sahle et al. 2017). Hence, previous reports describing the immunomodulatory mechanisms of MSCs may not have been able to detect SPM-related mechanisms easily because of the low level of the precursor PUFA availability. Although we observed SPM production in control-hBMSCs, which had been cultured in FBS-containing medium, the levels of produced SPMs may be low enough to render their effect difficult to identify in immunological assays. Moreover, understanding the

immunomodulatory mechanism of action of SPMs in MSCs would require conducting assays with specific inhibitors of biosynthesis or receptors in target cells. All in all, the impact of n-3 PUFA supplementation in cell culture medium and the possible boost in the proresolving response of hBMSCs requires further elucidation.

6.1.1 THE CHARACTERISTICS OF THE PHOSPHOLIPIDOME OF MSC-EVS

Even though MSC-EVs have received growing attention in MSC therapy due to their ability to induce a similar therapeutic response as the cells, their mechanisms of action are not fully elucidated (Varderidou-Minasian and Lorenowicz 2020). The lipid and LM metabolism has an important role in the MSC immunomodulation, thus, rendering it probable that EVs could mediate the lipid-related immunomodulatory effects of MSC. In Study II, we showed that the lipid metabolism of hBMSCs can be modified with PUFA supplementations. The changes may also be reflected as a changed lipidome in EVs as the content of cells typically resembles the content of EVs. Thus, we investigated the phospholipidome of hBMSC-EVs to determine if it was similarly modified with PUFA supplements. In this thesis, we studied hBMSC-EVs in general rather than dividing EVs into subcategories because of the difficulties in separating the exosomes and microvesicles from each other. Moreover, we were interested in the immunomodulatory effects of hBMSC-EVs in general, which further encouraged us not to separate EVs into small and medium/large fractions.

In contrast to the prior dynamics experiment, we first supplemented the hBMSCs with PUFAs for 24 hours to induce pronounced incorporation into the cell membranes. To collect a pure hBMSC-EV fraction, we next incubated the cells in serum-free medium for 48 hours and then isolated the EVs with ultracentrifugation. We determined the phospholipid profile of hBMSC-EVs and hBMSCs with ESI-MS/MS. The lipidome of hBMSC-EVs has previously been characterised but comparing our results with other studies is challenging due to the differences in the reporting formats (Vallabhaneni et al. 2015; Haraszti et al. 2016; Showalter et al. 2019).

Remarkably, we observed that the PUFA-modifications of the cells were transferred into the hBMSC-EV membranes. The accumulation of GP species with supplemented PUFAs and their elongated forms was clearly visible from the EV fraction. In general, the phospholipid profile of the EVs resembled the phospholipidome of the hBMSCs but certain changes were identified. When comparing the phospholipid species profiles of the EVs and cells, we observed that the lipid species in the EVs exhibited a similar trend to that seen in previous reports on EV phospholipids derived from other cell types (Skotland et al. 2017; Laurén et al. 2018; Valkonen et al. 2019). The previously identified accumulation of GP species with monounsaturated acyl chains, PS 18:0_18:1 in particular (Skotland et al. 2017), was also detected in our study. This is most

likely related to the optimal compatibility of these lipid species with the high curvature of the EV membrane.

Additionally, we observed the enrichment of di-PUFA GP species in EVs, which has also been reported in EVs derived from red blood cells and platelets (Laurén et al. 2018; Valkonen et al. 2019). The di-PUFA GPs were accumulated especially into the PUFA-supplemented hBMSC-EVs as the formation of di-PUFA GP species was also high in the corresponding cells. When the acyl chains of a phospholipid have a larger degree of unsaturation, the lipid becomes more mobile and the fluidity of the membrane increases. These factors most likely promote the efflux propensity and exit of the GPs from the plasma membrane during EVs budding. Moreover, the increase in chemical activity and efflux propensity causes the GPs to rise from the cell membrane more readily and become easily accessible substrates for different enzymes, for example for PLA_2 type IVA (Batchu et al. 2016). This PLA_2 enzyme cleaves especially polyunsaturated acyl chains from the *sn*-2 position of a GP releasing them for LM biosynthesis.

Intriguingly, EVs from different sources are known to transport SPMs and their pathway markers (Norling et al. 2011; Valkonen et al. 2019). Thus, the relative abundance of di-PUFA species in hBMSC-EVs may indicate that the EVs are efficient at LM and SPM biosynthesis due to the precursor availability or by providing the LM precursors to the recipient cells. However, the functional consequences of these PUFA modifications require further investigations.

6.2 ENHANCED ANTI-INFLAMMATORY PHENOTYPE OF REGULATORY MACROPHAGES BY MSCS AND THEIR EVS

Macrophages are versatile and highly plastic immune cells that have important functions in the homeostasis and inflammatory processes throughout the human body. The effect of MSCs on macrophages has received attention mainly from the point of view of M1 and M2 macrophages (described in detail in **Section 2.1.1.3**) but less is known of the impact of MSCs or MSC-EVs on the immunoregulatory Mregs. In Studies I and III, we inspected the effects of hBMSC cell-cell contact, secretome and EVs on the polarisation and phagocytosis activity of macrophages polarised and activated towards Mreg phenotype. In Study III, we further examined whether the effects of hBMSCs on these macrophages were potentiated by DHA and AA supplementations. A strict comparison of the results of the control-hBMSCs (no PUFA supplementation) are not directly applicable due to differences in the experimental setup of the Studies I and III. Mainly, the effect of hBMSC cell-cell contact and EVs were investigated in Study I, while we focused on the effect of hBMSC cell-cell contact and secretome in Study III. In addition to mature Mregs, i.e., Mreg-activated macrophages, we examined Mreg-

polarised cells in the macrophage assay in Study III. It is also important to note that the determined phenotype markers differed to a certain extent between the studies.

Remarkably, we were able to show that the hBMSC cell-cell contact, secretome and EVs all enhanced the anti-inflammatory phenotype of macrophages polarised and activated towards Mregs. We observed anti-inflammatory and proresolving changes in the LM and cytokine production, cell surface marker expression and phagocytosis activity of macrophages. As expected, there was variance in the phenotype of macrophages derived from different buffy coat donors. As the immune cells were all derived from different individuals, the variability is greater than that seen in experiments conducted utilising cell lines. Genetic, non-genetic and environmental factors, such as gender, age and annual seasonality, have been identified to affect the cytokine production of PBMCs in the large cohorts of the Human Functional Genomics Project (Li et al. 2016; ter Horst et al. 2016), and this variability is likely translated into other phenotype markers as well. Moreover, it has been reported that not all PBMC-derived immune cells or patients respond to the MSC treatment (non-responders), while certain people respond efficiently to MSCs (responders), reflecting the high variability detected in different individuals and emphasising the need for the standardisation of clinical MSC protocols (e.g., Giebel et al., 2017; Keto et al., 2018; Kurtzberg et al., 2020).

A solution to the high variability due to the utilisation of buffy coat-derived donors and variable responses to the macrophage polarisation would be to further increase the number of biological replicates. Unfortunately, in all of the studies this was not possible due to the demanding and laborious procedures of the macrophage assays and endpoint analyses. This was particularly true in Study III, where we investigated the effects of three hBMSC groups on two macrophage subtypes. In Study II, the low number of replicates and variability in especially the LM results is also a limitation of this study. However, the demanding experimentations and mass spectrometric analyses hindered the increasing of the number of replicates. Despite these challenges, we observed clear results in all the studies in this thesis.

6.2.1 LIPID MEDIATOR AND CYTOKINE LEVELS IN THE COCULTURE MEDIUM

Despite the individual variation, we observed clear effects of hBMSCs on the LM and cytokine production in the Mreg coculture medium. Remarkably, both hBMSC cell-cell contact and hBMSC-EVs increased the PGE_2 levels in the coculture medium with mature Mregs. MSCs are known to polarise macrophages towards an anti-inflammatory phenotype via PGE_2 (Németh et al. 2009; Ylöstalo et al. 2012; Chiossone et al. 2016; Jin et al. 2019) and, interestingly, PGE_2 can induce a suppressor phenotype in cultured macrophages (Broichhausen et al. 2012). These findings may indicate that the detected anti-inflammatory changes in our setting could be mediated by PGE_2.

Moreover, our results are consistent with previous studies investigating PGE_2 in the immunomodulation of MSC-EVs (Phinney et al. 2015; Harting et al. 2018). Moreover, the elevated PGE_2 levels by hBMSC-EVs suggests that the EVs may polarise macrophages via PGE_2.

Furthermore, the levels of 15-HETE and 17-HDHA were higher in the coculture medium of hBMSC cell-cell contact and Mregs when compared with Mregs alone. PGE_2 has been found to initiate LM class switching, during which the production of proinflammatory LTs is dampened, while the biosynthesis of proresolving lipoxins and 15-HETE are induced (Levy et al. 2001). The higher 15-HETE levels in the coculture of Mregs and hBMSCs may imply that the elevated concentration of PGE_2 acted as a cue for LM class switching. Overall, the PGE_2-induced LM class switching merits more studies on the functioning of MSCs. Actual SPM end-products were not detected in Study I, which most likely is due to the limits of detection of the LC-MS/MS system employed. Classical eicosanoids and monohydroxy pathway markers are more stable than SPMs, and we were able to detect them with our system. The analysis of SPMs, which are typically present in biological samples at low concentrations, is challenging, and the sensitivity of the methodology utilised was not sufficient for this analysis in Study I. On the contrary, in Study II, the LC-MS/MS method to detect LMs was very sensitive and therefore we were able to successfully determine the SPM profile of hBMSCs. Unfortunately, we could not investigate the LM profiles in Study III within the scope of this thesis due to the restrictions caused by the global COVID-19 pandemic.

Interestingly, both hBMSC cell-cell contact and hBMSC-EVs decreased IL-22 and IL-23 in coculture medium with mature Mregs in Study I. IL-22 and IL-23 are proinflammatory cytokines that are involved in the Th17 cell function by mediating Th17-produced inflammation and by maintaining Th17 effector population and pathogenicity, respectively (Bettelli et al. 2006; Zheng et al. 2007). The reduction of IL-23 could decrease the induction of pathogenic Th17 cells, and thus alleviate the pathogenicity of chronic diseases such as rheumatoid arthritis and Crohn's disease (Neurath 2017; Yago et al. 2017). We can therefore hypothesise that hBMSC cell-cell contact and hBMSC-EVs induced Mreg populations that may promote resolution by reducing Th17 conversion or Th17 pathogenicity. IL-23 production was measured also in Study III, however, the levels of IL-23 were not reduced by hBMSC cell-cell contact. Overall, the levels of IL-23 were at the detection limits of the assay, which could be due to buffy coat donor-related differences, a slightly changed experimental setting or different analysis methods employed in these studies (Luminex-based ProcartaPlex Immunoassay in Study I and ELISA in Study III).

6.2.2 CHANGES IN THE CELL-SURFACE PHENOTYPE MARKERS OF REGULATORY MACROPHAGES

Different hBMSC conditions altered the expression of the cell surface phenotype markers of macrophages. In both studies, the cell surface markers CD86, CD206 and CD163 were investigated, while the rest of the markers discussed were examined only in one study.

The different hBMSC conditions had a limited effect on the expression of CD80 and CD86, which are cofactors required for T-cell activation. Mregs express CD80 at low levels (Hutchinson et al. 2011) as observed in Study I. The hBMSC cell-cell contact decreased this low expression even further, indicating an augmented suppressor capacity. Generally, Mregs are positive for CD86 (Hutchinson et al. 2011) as observed in our reports. The only change in the CD86 expression was a slight increase induced by hBMSC-EVs. This finding may indicate that these Mregs have a more activated phenotype. However, the overall CD86 expression levels were variable.

In general, the polarisation of macrophages is demanding and can be affected by various buffy coat donor-related factors, as discussed in the **Section 6.2**, and by experimental factors. For example, the experiments in Study I were conducted utilising 24-well plates, but in Study III with 12-well plates in order to increase the surface area of the insert for hBMSC culture. Moreover, the reagents, such as growth factors and FBS, which may have high batch-to-batch variability, had changed between the studies. This may have had an unexpectedly large effect in cell culture and cell polarisation (van der Valk et al. 2018). All of these factors may underlie behind the observed discrepancies in the cell surface phenotype markers and cytokine profile. Moreover, although the secretome contains EVs, the experimental settings between the secretome and EVs in our studies are not directly comparable. Previously, the EVs added to Mregs were derived from unstimulated hBMSCs and given in two doses. In the current study, the hBMSCs constantly produced EVs together with other soluble factors in stimulated environment with macrophage coculture.

The most drastic effect on cell surface markers was induced by the hBMSC secretome, which elevated the expression of mannose receptor CD206 in Mreg-polarised macrophages and mature Mregs in Study III. CD206 mediates the phagocytosis of microbes by binding to the carbohydrate residues of glycans on their cell surface (Stahl and Ezekowitz 1998). Both human and murine MSCs and their EVs have been shown to increase the CD206 expression of monocytes or macrophages polarised towards M1-phenotype (Kim and Hematti 2009; Zhang et al. 2010; Ylöstalo et al. 2012; Chiossone et al. 2016; Henao Agudelo et al. 2017; Lo Sicco et al. 2017). In our study, we observed the same response in Mreg-polarised macrophages and mature Mregs. The hBMSC cell-cell contact had no effect on CD206 nor haemoglobin scavenger receptor CD163 expression in Study III, however, both hBMSC cell-cell contact and hBMSC-EVs decreased CD206 and CD163 expression in Study I. CD206 and CD163 are typical markers of the alternatively activated M2-

macrophages involved in phagocytosis and limitation of inflammation, respectively (Mosser and Edwards 2008; Etzerodt and Moestrup 2013). These differing results may indicate that the hBMSC secretome polarises Mregs towards M2-phenotype, while EVs alone and the cell-cell contact have a differing impact, perhaps by promoting the immunoregulatory role of suppressor Mregs rather than by improving phagocytosis and attenuating inflammation.

In addition to CD206 expression, the hBMSC secretome elevated the expression of PD-L1 in Mreg-polarised macrophages and MerTK in mature Mregs. PD-L1 binds to a co-inhibitory receptor programmed death 1, which causes the inhibition of T-cell activation and proliferation leading to an attenuated immune response (Sun et al. 2018). Our observation is supported by a previous study, where MSCs elevated the PD-L1 expression of M2-macrophages (Abumaree et al. 2013). Interestingly, PGE_2 can also increase the PD-L1 expression in macrophages (Prima et al. 2017), which may be related to the improved PD-L1 expression in our study. Thus, MSCs render the phenotype of macrophages more proresolving as PD-L1 is expressed on the cell surface of resolution-phase macrophages (Shouval et al. 2014; Wynn and Vannella 2016).

We observed another proresolving phenotype change when the hBMSC secretome increased MerTK expression in mature Mregs. MerTK is an established marker for antifibrotic M2c-macrophages (Zizzo et al. 2012). Moreover, MerTK is involved in the clearance of apoptotic cells (Scott et al. 2001), SPM production (Cai et al. 2016) and promotion of resolution of inflammation in acute liver injury (Triantafyllou et al. 2018), highlighting the pivotal role of this enzyme in the resolution of inflammation. Intriguingly, it has been reported that EVs from cardiosphere-derived cells are able to elevate the MerTK expression of macrophages via the transfer of microRNA-26a (De Couto et al. 2019), which may indicate that the EVs in the hBMSC secretome are mediating the elevated MerTK expression.

The hBMSC cell-cell contact had a smaller impact on the macrophage phenotype than the secretome in Study III by only decreasing HLA-DR expression. HLA-DR is a proinflammatory marker and is involved in the activation of T cells via antigen presentation. In agreement with our results, previous reports have described that MSCs diminish the HLA-DR expression of macrophages and monocytes (Hanson et al. 2011; Wise et al. 2016; Hong et al. 2019; Islam et al. 2019). To summarise, the effect of the hBMSC secretome was greater than the cell-cell contact in modifying the cell surface markers. The secretome skewed the macrophages towards a more anti-inflammatory and proresolving phenotype by elevating CD206, PD-L1 and MerTK expression. In general, soluble factors and EVs mediate the majority of the MSC-derived effects (Giebel et al. 2017; Volarevic et al. 2017). Although the hBMSCs in cell-cell contact also produce soluble factors and EVs, their effect did not manifest at the same level as with the hBMSCs grown on inserts.

6.2.3 PHAGOCYTOSIS ACTIVITY OF MACROPHAGES

In Study III, we investigated whether CD206 expression induced by the hBMSC secretome increased the phagocytosis activity of macrophages. CD206 is a pattern recognition receptor that recognises and binds to the mannose residues on the cell surface of *C. albicans* (Maródi et al. 1991; Stahl and Ezekowitz 1998). Therefore, we utilised fluorescent-labelled *C. albicans* to determine the phagocytosis activity of Mreg-polarised macrophages. In contrast to our earlier results, not all PBMC-derived macrophages responded to the hBMSC secretome by increasing CD206 expression. However, in the ones that responded, both the CD206 expression and the phagocytosis activity were increased by the hBMSC secretome.

Previously, both murine and human MSCs have been shown to induce the phagocytosis of macrophages in various assays employing different yeast or bacteria species or artificial beads (Abumaree et al. 2013; Hegyi et al. 2014; Deng et al. 2015; Jackson et al. 2016; Kudlik et al. 2016). Thus, our novel finding that hBMSCs induce the phagocytosis of *C. albicans* by macrophages is in line with these previous studies employing other assays. One of the key aspects in the resolution of inflammation is the clearance of pathogens and apoptotic cells via phagocytosis and efferocytosis, respectively (Buckley et al. 2014). Phagocytosing macrophages become more prevalent during resolution of inflammation when the cell debris and microbes are cleared away to achieve homeostasis (Wynn and Vannella 2016), emphasising the ability of hBMSC secretome to promote the proresolving phenotype of macrophages. In future studies, it would be interesting to investigate the efferocytosis activity of MSC-primed macrophages by utilising apoptotic neutrophils to determine the impact of MSCs on this important phenomenon in resolution.

In Study I, we did not detect an improvement in the phagocytosis activity of Mregs by hBMSC cell-cell contact. However, we utilised IgG-coated latex beads, which are bound by opsonic Fcγ receptor in contrast to the non-opsonic receptors, i.e., CD206 (Bournazos and Ravetch 2017). Thus, the mechanisms of *C. albicans* and IgG-mediated phagocytosis were different as were the examined paracrine and contact-based effects of hBMSCs (i.e., secretome in Study III and cell-cell contact in Study I), leading to differing results between these studies.

All in all, we observed multiple effects of hBMSCs and hBMSC-EVs on macrophages polarised towards Mreg-phenotype that included changes in the phagocytosis activity, cell surface protein expression, cytokine and LM production. As Mregs themselves are immunoregulatory cells and already possess a tolerogenic phenotype, the effects of hBMSCs on their phenotype were less pronounced than in the case of macrophages polarised towards host defence M1-phenotype, which elicit proinflammatory actions. Overall, the hBMSCs and hBMSC-EVs promoted the anti-inflammatory and proresolving phenotype of these less-studied immunoregulatory macrophages even further.

6.2.4 EFFECT OF PUFA SUPPLEMENTATION ON THE IMMUNOMODULATORY POTENTIAL OF MSCS

The lipid metabolism of MSCs has an important role in the immunomodulatory response. Interestingly, the n-6/n-3 PUFA ratio of the cell membranes correlates with the immunosuppressive capacity of hBMSCs: improved suppression of T-cell proliferation was observed in cells with more n-3 PUFAs in their membranes (Kilpinen et al. 2013b). These findings led us to wonder whether modifying the membrane n-6/n-3 PUFA ratio favouring n-3 PUFAs would have an impact on the immunomodulation of human MSCs. This hypothesis is also supported by previous studies on n-3 PUFA-supplemented MSCs (Tsoyi et al. 2016; Abreu et al. 2018; Mathew and Bhonde 2018; Silva et al. 2019).

By examining the effects of PUFA supplementation on the phospholipid, fatty acid and LM profiles in Study II, we observed profound changes in the lipidome of hBMSCs and hBMSC-EVs. Remarkably, the modifications in the phospholipid profile were transferred into hBMSC-EVs indicating that the lipid metabolism of the EVs was to a great extent affected by the supplementation. In Study III, we next determined the functional effects of these PUFA-modifications on hBMSC cell-cell contact and secretome in the Mreg polarisation assay by supplementing the cells with DHA or AA. Before investigating the PUFA-supplemented EVs directly, we examined only PUFA-supplemented cells in these experiments. This allowed us to assess the suitability of the culture conditions for elucidating the effects of PUFA supplementations *in vitro* and to consider possible changes for future experiments.

Interestingly, the secretome of DHA-hBMSCs induced the most potent anti-inflammatory phenotype in Mreg-polarised macrophages, while the mature Mregs remained unaffected. The DHA-hBMSC secretome induced an elevated expression of CD163 and a reduced gene expression of MMP-2. The haemoglobin scavenger receptor CD163 is expressed on the alternatively activated M2-macrophages and is known to limit inflammation (Etzerodt and Moestrup 2013). In contrast, MMP-2, which is secreted by M1 macrophages, degrades the extracellular matrix and increases the recruitment of inflammatory cells (Parks et al. 2004). Our results are in line with previous studies demonstrating that MSCs decrease the macrophage gene expression of MMP-2 (Hashizume et al. 2011; Yamawaki-Ogata et al. 2014; Lin et al. 2020), emphasising the ameliorated immunophenotype of DHA-hBMSC secretome. According to Study II, DHA supplementation augments the production of DHA-derived SPMs RvD4 and RvD6, which could mediate the improved immunomodulatory actions of DHA-hBMSCs.

Although the DHA-hBMSCs elicited the most potent anti-inflammatory actions, the effects of control- and AA-hBMSCs were similar to those of the DHA-hBMSCs. Consequently, by utilising a macrophage assay with the polarisation of PBMCs towards an Mreg phenotype, we observed only limited changes in the immunomodulation of hBMSC secretome by PUFA

supplementation. However, previous studies with EPA- and DHA-supplemented MSCs have indicated that the immunomodulation of MSCs can be enhanced with PUFA supplementation in *in vivo* settings (Tsoyi et al. 2016; Abreu et al. 2018; Silva et al. 2019). Moreover, a previous study supplementing human MSCs with a fatty acid mixture mimicking the *in vivo* fatty acid composition resulted in an improved immunosuppressive capacity of MSCs in an *in vitro* PBMC assay (Chatgilialoglu et al. 2017). A detailed composition of the mixture was not reported but it was indicated that it contained multiple n-6 PUFAs. Overall, a mixture of PUFAs may be more effective in modifying the SPM composition than a single PUFA. It has been reported that a combination of PUFAs, with the emphasis on n-3 PUFAs, ameliorates airway inflammation in asthmatic mice more effectively than EPA supplementation alone through better counter-regulation of the formation of proinflammatory LMs and maximisation of the SPM pool (Fussbroich et al. 2019, 2020).

Due to the global COVID-19 pandemic, we were not able to investigate the impact of PUFA-supplemented hBMSCs on LM production in the macrophage coculture in this thesis. The LM profile might have responded more strongly to the PUFA-modified membranes than the cell surface phenotype markers or cytokine profiles. The PUFA-modified cell membranes likely affect the immune cells by secreting LMs into the coculture medium and also by secreting PUFA-modified EVs, as suggested by the results of Study II. Unfortunately, our attempt to profile the SPM composition of hBMSC-EVs was also prevented by the pandemic.

Moreover, we studied the effects of DHA and AA in the macrophage assay. In the light of two recent publications employing EPA-MSCs, it would have been interesting to investigate the effects of EPA-hBMSCs also in our setting (Abreu et al. 2018; Silva et al. 2019). As AA-derived PGE_2 plays a pivotal role in the MSC-immunomodulation, the corresponding EPA-derived molecule, PGE_3, could also be of significance in mediating the anti-inflammatory response of MSC. In the studies by Abreu (2018), Silva (2019) and colleagues, it was reported that the EPA-MSCs produced more PGE_2 than control MSCs. However, because the levels of PGE_2 were measured with an ELISA kit with over 40% cross reactivity to PGE_3, it cannot be ruled out that this EPA-derived PG may play a role in the observed enhanced therapeutic effect. Unfortunately, we could not determine the PGE_3 production by hBMSCs in Study II because it was not included in the analysis panel. However, it would be very important to assess this LM in the future because PGE_3 has similar effects to PGE_2, even though less proinflammatory (Schmitz and Ecker 2008), and its role in MSC immunomodulation is unknown. It is also important to note that supplementing hBMSCs with EPA increased the membrane levels of two SPM precursors: EPA and n-3 DPA. Thus, PGE_3, EPA- and n-DPA-derived SPM signalling could all contribute to the observed efficacy of EPA-MSCs in *in vivo* models (Abreu et al. 2018; Silva et al. 2019).

The results of Study III indicate that our experimental macrophage *in vitro* setting may not have been an optimal method for pinpointing the effects of

PUFA supplementations on hBMSC immunomodulation. It may be that the PUFA-mediated immunomodulation is of less significance when hBMSCs modulate immunoregulatory macrophages. Moreover, *in vitro* models may emphasise the role of specific mechanisms of action. For example, the suppression of T-cell proliferation is largely mediated by IDO in *in vitro* assays (Meisel et al. 2004; François et al. 2012). Additionally, the significance of PGE_2 has been established in the MSC-mediated modulation of macrophages (Németh et al. 2009; Ylöstalo et al. 2012; Chiossone et al. 2016; Jin et al. 2019). It may be that PGE_2-mediated signalling dominates also in our macrophage assay, rendering it more difficult to detect SPM-mediated changes. All in all, the role of SPMs in the functionality of human MSCs is less studied (Fang et al. 2015) and the complete unravelling of a novel SPM-mechanism requires further investigations.

Even though we did not observe drastic changes in the immunomodulatory phenotype of PUFA-modified hBMSCs, we firmly believe that PUFA supplementation, and lipid metabolism in general, has a pivotal role in the immunomodulation of hBMSCs. This hypothesis is supported by our results with the DHA-hBMSCs and multiple reports of others investigating the immunomodulation of PUFA-modified MSCs (Tsoyi et al. 2016; Chatgilialoglu et al. 2017; Abreu et al. 2018; Mathew and Bhonde 2018; Silva et al. 2019). Further studies are required to identify the ideal experimental settings and cell types for elucidating the role of PUFA-mediated hBMSC immunomodulation.

7 CONCLUSIONS AND FUTURE PROSPECTIVES

This thesis provides novel and fascinating insights into the immunomodulation of hBMSCs from the point of view of proresolving LMs and the secreted EVs. Our focus was on the PUFA supplementation, which could be employed to improve the immunomodulatory response of hBMSCs. The main contribution of this thesis to the MSC biology is the provision of further evidence for the importance of the PUFA-mediated immunomodulation of hBMSCs, in which novel proresolving LMs play a key role. Moreover, we have emphasised the role of EVs in mediating the anti-inflammatory response of the hBMSCs and demonstrated that EVs can be modified with PUFA supplementations to the cells. Importantly, we have elucidated the effects of hBMSCs and hBMSC-EVs to Mregs, which had not been investigated prior to our reports.

In this thesis, we examined primary hBMSCs derived from different donors and their effects on human PBMC-derived macrophages, which were likewise derived from multiple voluntary donors. Research *in vitro* has its limitations; however, the utilisation of primary human cells provides evident advantages over the use of immortalised cell lines or murine cells in the applicability of the results to human health.

We were able to show that hBMSCs and hBMSC-EVs enhance the anti-inflammatory and proresolving properties of the suppressor macrophages, Mregs. The effects of hBMSCs and hBMSC-EVs on Mregs were comparable to the changes determined previously in M1 macrophages. This indicates that both the cells and their EVs are able to promote the polarisation of macrophages towards anti-inflammatory phenotype, thereby promoting the dampening of immune responses. The observation that hBMSC-EVs promoted the anti-inflammatory phenotype of Mregs supports the idea of applying MSC-EVs as therapeutic agents.

Moreover, we showed that the phospholipid, fatty acid and LM profiles of hBMSCs are profoundly modified with PUFA supplementation. For the first time, we determined the SPM profile of hBMSCs providing further evidence that these highly potent proresolving molecules have a role in the MSC functionality. Additionally, the LM profiles reflected the membrane PUFA content by increasing the downstream LM production with elevated precursor PUFA availability. The modifications of the cell membranes were transferred into the membranes of hBMSC-EVs, indicating that the LM metabolism of the EVs may have been affected as well. As n-3 PUFA-modified MSCs have been reported to elicit an improved immunomodulatory phenotype, the altered LM, and especially the SPM, profiles are likely to mediate the improved therapeutic potential of MSCs.

We further examined the effects of PUFA-modified hBMSCs in the Mreg-assay, where we observed a potentiated immunophenotype of hBMSCs supplemented with DHA that could be derived from the increased production of proresolving RvDs. Nevertheless, the PUFA modifications induced only a limited improvement in the hBMSC functionality. The investigation of PUFA-modified MSCs should be extended into other immunological *in vitro* and *in vivo* assays. Previous promising results on n-3 PUFA-modified MSCs have been reported in murine *in vivo* models. Finding an effective *in vitro* model utilising human cells would a great asset when investigating the effects of PUFA-modified MSCs prior to giving the cells to patients. Although the inspection of the functionality of PUFA-modified EVs was not within the scope of this thesis, it would be highly interesting to see whether the PUFA-mediated improvement is induced by EVs and also to determine their SPM profile.

Priming of MSCs with different supplemented molecules and changed culture conditions has intrigued researchers for a long time and represents a feasible way to improve the therapeutic potential of MSCs (Noronha et al. 2019). PUFA supplementation would represent an easy and safe way to prime clinical MSCs. Dietary PUFA supplements have been safely consumed for a long time and supplementing cells with PUFAs *in vitro* can be compared with changing the serum composition or platelet-rich plasma donor or batch. Nevertheless, the safety and efficacy of PUFA-modified MSCs should be carefully elucidated before implementing them in the clinic. The exact culture conditions, including the concentration and length of the supplementation, require further studies. Moreover, a mixture of PUFAs developed to maximise the SPM pool in cells should be considered as an alternative to using a single PUFA. All in all, improving the immunomodulatory capacity and standardisation of MSC and the future MSC-EV therapies are in the key position when aiming for the most effective therapeutic response and reproducibility of these therapies.

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